SEQUENTIAL DELIVERY OF ANTIBIOTICS AND PROBIOTICS EMPLOYING A DUAL RELEASE MECHANISM

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A thesis submitted to the Faculty of Health Sciences, University of the

Witwatersrand, in fulfillment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I, Mershen Govender, declare that this thesis is my own work. It has been submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences at the University of Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.

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This.....day of December 2014

RESEARCH OUTPUTS

Publications

1. Mershen Govender, Yahya E. Choonara, Pradeep Kumar, Lisa C. du Toit, Sandy van Vuuren, Viness Pillay. Advancements in probiotic delivery: Conventional vs. Non-conventional formulations for intestinal flora supplementation, *AAPS PharmSciTech*, 2014, 15(1), 29-43. (Appendix A1).

2. Mershen Govender, Yahya E. Choonara, Sandy van Vuuren, Pradeep Kumar, Lisa C. du Toit, Viness Pillay. A gastro-resistant ovalbumin mini-tablet-in-tablet system for the delivery of *Lactobacillus acidophilus* probiotic to simulated human intestinal and colon conditions. (Submitted to *Journal of Pharmacy and Pharmacology*, March 2014). (Appendix A2).

3. Mershen Govender, Yahya E. Choonara, Sandy van Vuuren, Pradeep Kumar, Lisa C. du Toit, Lomas K. Tomar, Charu Tyagi, Viness Pillay. A modified MRS agar for the selective enumeration of *Lactobacilli* in aspirated porcine intestinal fluid. (Submitted to *Letters in Applied Microbiology*, November 2014). (Appendix A3).

4. Mershen Govender, Yahya E. Choonara, Sandy van Vuuren, Pradeep Kumar, Lisa C. du Toit, Lomas K. Tomar, Charu Tyagi, Viness Pillay. An oral multi-particulate system for the concurrent delivery of amoxicillin and *Lactobacillus acidophilus* probiotic. (To be submitted to *Future Microbiology*). (Appendix A4).

5. Mershen Govender, Yahya E. Choonara, Sandy van Vuuren, Pradeep Kumar, Lisa C. du Toit, Lomas K. Tomar, Charu Tyagi, Kennedy Erlwanger, Viness Pillay. *In vitro*/*ex vivo*/*in vivo/in silico* evaluation of a dual release system for the concurrent delivery of antibiotics and probiotics. (To be submitted to *Pharmaceutical Research*).

Conference Proceedings

1. Mershen Govender, Viness Pillay, Yahya E. Choonara, Lisa C. du Toit, Sandy F. van Vuuren. A multi-particulate system for the concurrent delivery of amoxicillin antibiotic and *Lactobacillus acidophilus* probiotic, University of Witwatersrand 2nd Cross-Faculty Research Symposium, 22 October 2012. (Appendix B1).

2. Mershen Govender, Yahya E. Choonara, Sandy F. van Vuuren, Pradeep Kumar, Lisa C. du Toit, Lomas K. Tomar, Charu Tyagi, Viness Pillay. Fabrication, Statistical Optimization and Characterization of a Multi-particulate System for the Concurrent Delivery of Antibiotics and Probiotics, University of Witwatersrand School of Therapeutic Sciences Research Symposium, 10 September 2013. (Appendix B2)

3. Mershen Govender, Yahya E. Choonara, Sandy F. van Vuuren, Pradeep Kumar, Lisa C. du Toit, Lomas K. Tomar, Charu Tyagi, Viness Pillay. *In vivo* analysis of a concurrent delivery system for antibiotics and probiotics in a Large White pig model. University of Witwatersrand Faculty of Health Sciences Research Day, 17 September 2014. (Appendix B3)

PATENTS

Mershen Govender, Viness Pillay, Yahya E. Choonara, Pradeep Kumar, Lisa C. du Toit, Probiotic drug delivery system. SA Provisional Patent Application: 2013/03979.

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ABSTRACT

Antibiotic therapy has been proven to be vital for the treatment of life-threatening bacterial infections. Oral antibiotic therapy, however, results in unwanted side effects such as the intestinal flora destruction, allowing for the colonization of foreign bacteria. This phenomenon results in the occurrence of antibiotic-associated diarrhea. Probiotic supplementation has been the choice adjunctive prophylaxis for this condition allowing for the bacterial adhesion of intestinal mucosal binding sites. Probiotic bacteria are, however, susceptible to the bactericidal effects of broad-spectrum antibiotics, resulting in many probiotic formulations being prescribed two hours after the ingestion of the antibiotic formulation. This is, however, not always adhered to, with many patients taking the antibiotic and probiotic concomitantly resulting in the destruction of the probiotic bacteria. This study provides for the design, development, characterization and evaluation of an oral delivery system for the concurrent administration of antibiotics and probiotics employing a dual release mechanism or 'Dual-Biotic System'. The premise behind the development of this system is to allow for the concurrent administration of antibiotics and probiotics where the probiotic bacteria are only released two hours after the antibiotic, in which time the antibiotic would be absorbed into systemic circulation, preventing physical interaction between the systems and thus preventing bacterial destruction. Amoxicillin was chosen as the model antibiotic in this study due to its spectrum of activity and wide utilization in oral antibiotic therapy.

The designed Dual-Biotic System consists of an active biopolymeric granule system derived from raw egg ovalbumin that allows for the protection and controlled delivery of probiotic *Lactobacillus acidophilus*. This ovalbumin component has been denatured and crosslinked as part of the formulation process, utilizing phosphate buffer saline and genipin respectively, with the ovalbumin matrix statistically optimized utilizing a Box-Behnken experimental design to set therapeutic and formulation criteria. Preformulation analyses were conducted on the ovalbumin matrix to ascertain its probiotic release properties in addition to its susceptibility to protein degradation. Molecular docking studies were also undertaken and determined the mechanism of probiotic release from the ovalbumin matrix in addition to the crosslinking effects of genipin on the protein structure. Statistical optimization determined that the optimized ovalbumin formulation composed of 0.8868mL ovalbumin, 0.0058% genipin and a buffer pH of 6.9929. This system was thereafter advanced into a delayed release system composed of glyceryl monostereate at a ratio of 1:1.94 that allowed for a two delay in the release of the probiotic bacteria. The Dual-Biotic system was formulated by incorporating the delayed release granules and amoxicillin trihydrate within a gastro-resistant hard gelatin capsule for targeted intestinal delivery.

Advanced *in vitro* and *ex vivo* characterization analyses were thereafter undertaken on the optimized, delayed release and Dual-Biotic systems, with stability analyses performed over a three month period detailing increased bacterial survival from the respective systems. *Ex vivo* adhesion studies were also undertaken in which the high adhesion potential of *L. acidophilus* was noted, in addition to its ability to resist bacterial adhesion of two commonly occurring pathogenic bacteria, *Escherichia coli* and *Clostridium difficile*. *In vivo* analysis of the Dual-Biotic System utilizing a Large White pig model determined the effectiveness of the system to protect and deliver *L. acidophilus* probiotic bacteria concurrently with amoxicillin. As part of the *in vivo* study, a stable cannula was designed and surgically implanted into the ileum of the pig for intestinal fluid aspiration, with samples inoculated onto a developed modified MRS agar for the selective enumeration of *Lactobacilli*. Pharmacokinetic modeling determined that amoxicillin and *L. acidophilus* release was best described by a one compartmental model with lag with a Level-A *in vitro-in vivo* correlation developed detailing a 96.6% predictability of amoxicillin release. It was therefore concluded that the developed Dual-Biotic System successfully delivered amoxicillin and *L. acidophilus* concurrently with a high *in vitro-in vivo* correlation determined in addition to high bacterial stability. Results determined therefore confirmed the possibility of the system to be utilized for the prevention of antibiotic-associated whilst ensuring patient compliance through the administration of a single dosage form.

DEDICATION

To my parents, Tilo and Kogie Govender, who have shown me that through hard work dedication and belief, anything is possible. For all the love, sacrifice, support and encouragement that you have shown me in the completion of my postgraduate studies, I dedicate this thesis to you.

"If you are to do anything, give it your best, do it once, do it right, life is too short to come back and try again" – words to live by given to me by my mother, Mrs K Govender.

ANIMAL ETHICS DECLARATION

I hereby confirm that the following study entitled "*In vivo* analysis of an oral multi-particulate system derived from ovalbumin for the concurrent delivery of amoxicillin and *Lactobacillus acidophilus* probiotic in a pig model" had received approval from the Animal Ethics Committee of the University of the Witwatersrand with ethics clearance number 2012/26/05.

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LIST OF COMMONLY USED ABBREVIATIONS

AoR- Angle of Repose

ATP- Adenosine triphosphate

BMTTS- Bi-layered minitablet-in-tablet system

CCI- Carr's Compressibility Index

CFUs- Colony Forming Units

FTIR- Fourier Transform Infrared Spectroscopy

IVIVC- *In vitro-in vivo* correlation

LA- *L. acidophilus*

LP- *Lactobacillus* probiotic

MRI- Magnetic Resonance Imaging

MPC- Minimum probiotic concentration

MPCT- Minimum probiotic concentration time

MRS - de Man, Rogosa and Sharpe

SGF- Simulated gastric fluid

SIF - Simulated intestinal fluid

TPC- Target probiotic concentration

TSA- Tryptone Soya agar

CHAPTER 1
INTRODUCTION

1.1 Background to this Study

The human gastrointestinal tract contains over 500 microbial species and includes approximately 10^{14} functional bacterial cells in addition to various fungi, yeasts, viruses and protozoa (Iannitti and Palmieri, 2010). These gastrointestinal flora have been shown to be a vital part of the intestinal health of humans by providing protection against foreign pathogenic bacteria as well as aiding in the digestion of food products. Some of these bacteria, however, such as *Streptococci* and *Staphylococci* spp., have been known to cause opportunistic infectious diseases in humans (Kopp-Hoolihan, 2001; Lourens-Hattingh and Viljoen, 2001; Del Piano *et al*., 2006; Stevenson and Blaauw, 2011). The constituent of intestinal flora varies from patient to patient with many factors such as age, race and geographical location influencing the species and number of bacteria found in the gastrointestinal tract (Brown *et al*., 2012). The health benefits of gastrointestinal flora, however, do remain constant despite patient variations. A full representation of the typical microbial content of the human gastrointestinal tract is shown in Figure 1.1.

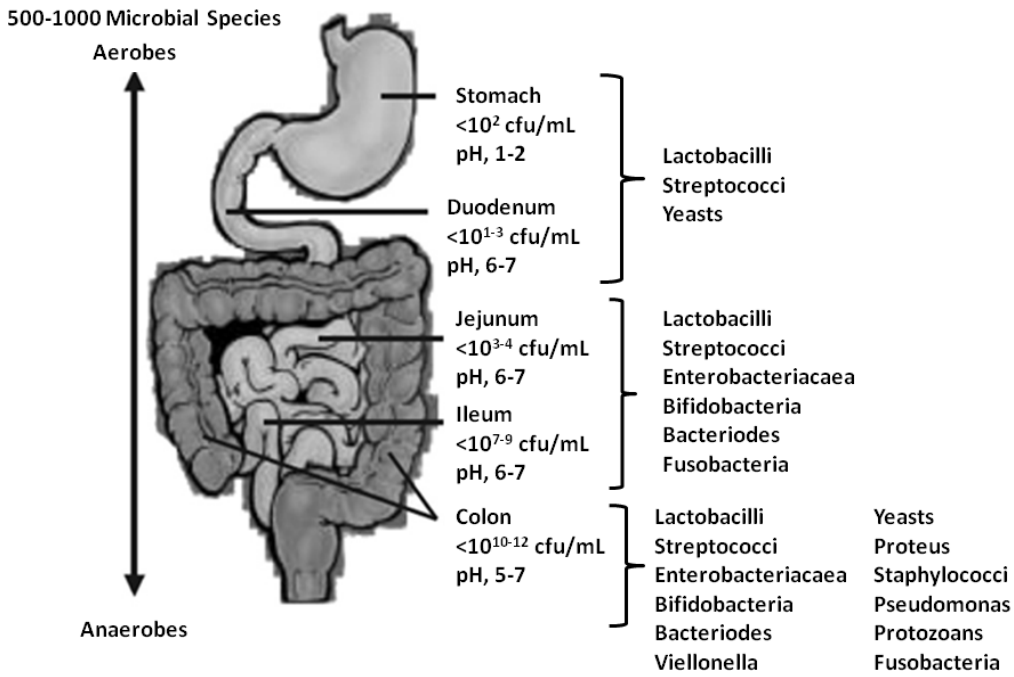


Figure 1.1: Summary of the microbial content found in a healthy human gastrointestinal tract. Image adapted from Iannitti and Palmieri (2010), Microbial species adapted from Holzapfel *et al*. (1998).

1.1.1 Gastrointestinal flora depletion

Gastrointestinal flora are depleted for various reasons ranging from stress to infection, antibiotic use, dietary changes and environmental factors (Iannitti and Palmieri, 2010). This often leads to opportunistic infections by both native and foreign pathogenic bacteria. From a clinical perspective, the most important cause of intestinal flora depreciation is by oral antibiotic use, primarily through the use of broad-spectrum antibiotics, which by their mechanism of action destroy both healthy and pathogenic bacteria (Katzung, 2007; Kohanski *et al*., 2007). This decrease in intestinal flora composition as a result of antibiotic use leads to the pathogenic invasion of foreign bacteria, such as *Clostridium difficile*, the major contributor to antibiotic-associated diarrhea (Wenisch *et al*., 1996; Iizuka *et al*., 2004; McFarland, 2006).

Probiotic bacteria are living supplementary organisms that replenish lost natural intestinal flora, thereby providing protection against foreign pathogenic infections, as well as providing other health benefits to the host (Fuller, 1991; FAO-WHO, 2002; Saarela *et al*., 2002; Collado *et al*., 2007; Bosch *et al*., 2011). Patients that often require probiotic supplementation are immuno-compromised individuals such as those undergoing oral antibiotic therapy utilizing broad-spectrum bactericidal drugs. Probiotic supplementation in these patients has been shown to be vital in the prevention of potentially fatal, pathological infections (Todd, 2003; Kuitunen *et al*., 2009; Iannitti and Palmieri, 2010). Other patients that require probiotic therapy are babies born of caesarean section and do not gain the bacteria it would naturally whilst travelling down the birth canal (Stevenson and Blaauw, 2011).

In addition to intestinal flora supplementation, probiotics have also been found through various studies to have favorable clinical health effects in the treatment of Crohn's disease, cancer, irritable bowel disease, constipation and many other gastrointestinal conditions (Iannitti and Palmieri, 2010). Probiotic bacteria have also been widely recognized to have other health benefits to the patient such as positive effects on immunological functions, aiding in digestion and protection against pathogenic bacteria such as *Salmonella typhimurium*, *Helicobacter pylori* and *Escherichia coli* (Juntunen *et al*., 2001; Janer *et al*., 2004; Penner *et al*., 2005; Sunada *et al*., 2008; Iannitti and Palmieri, 2010). Other functions include the improvement of lactose intolerance, decreasing cholesterol levels and the treatment of ulcerative colitis (Mitsuoka, 1982; Hove *et al*., 1999; D'Souza *et al*., 2002; McConnell *et al*., 2008; Yamada *et al*., 2008; Reiff and Kelly, 2010).

1.1.2 Concomitant antibiotic and probiotic administration

Probiotic bacteria are most often utilized as prophylactic treatment to antibiotic-associated diarrhea during antibiotic therapy and is generally prescribed to be taken approximately two hours after the oral administration of the antibiotic product (Hendriks and Duggan, 2005; Rakel, 2012). This, however, leaves the requirement of patient compliance, with the concomitant administration of the probiotic with the antibiotic by the patient resulting in the therapeutic failure of the probiotic. This consequently results in the onset of antibiotic-associated diarrhea (Hempel *et al*., 2012). With the issue of concomitant delivery of antibiotics and probiotics documented, the use of probiotic delivery systems containing yeasts which are not naturally part of the gastrointestinal tract has occurred (Foligne *et al*., 2010). These yeasts function in the competition of mucosal adhesion sites, with a few species having antibacterial actions through the secretion of antibacterial agents. Probiotic bacteria are usually naturally occurring as part of the gastrointestinal system and have the ability to perform the functional health benefits associated with natural gastrointestinal flora (Iannitti and Palmieri, 2010). The use of naturally occurring bacteria, when administered separately to antibiotics would, therefore, ultimately provide greater health benefits to the patient when compared to probiotics utilizing unsusceptible yeasts. There is therefore a need for the development of a delivery system that would protect probiotic bacteria against the bactericidal effects of antibiotics whilst ensuring patient compliance through the administration of a single dosage form.

The proposed concurrent delivery system was achieved through the development of a delayed release system that would allow for the absorption of the delivered antibiotic prior to the release of the probiotic bacteria in the GIT. The delayed release system consisted of a protective carrier matrix for the probiotic, ovalbumin, which would ensure bacterial survivability during the formulation process. Ovalbumin, the major component of chicken egg albumin, is a cheap commonly found protein that can be used for the delivery of actives such as probiotic bacteria. This is as a result of the probiotic bacteria being incorporated into the protein matrix, thereby providing protection to the microorganisms against external forces such as compression and large changes in temperature, which is detrimental to the survival of probiotic bacteria (Panigrahi *et al*., 2007). Further advantages of utilizing ovalbumin is its ability to withstand gastric acid degradation, providing gastric protection for the probiotic bacteria and furthermore has the ability to provide a growth enhancing media upon digestion, promoting bacterial growth after delivery (Martos *et al*., 2010). Denaturation of a protein such as ovalbumin through chemical, thermal or mechanical processes has also been shown to alter both the physical and chemical properties of the protein structure. This

resultantly influences the release properties of the protein and can be utilized to modify the protein structure to the requirements of a delivery system (Cooper, 1999; Matsudomi *et al*., 2001; Ophardt, 2003). Therefore, by exposing the ovalbumin structure to chemical denaturation, the ideal denatured protein structure was obtained for the delivery of probiotic bacteria. The delayed release component was formulated utilizing a hydrophobic erodible system, glyceryl monostereate, which prevented premature release of probiotic bacteria, whilst also allowing for particulate aggregation *in vivo*, decreasing intestinal transit time, and thereby allowing probiotic release in the proximal intestinal tract.

This study therefore provides for the design and formulation of an ovalbumin granule delivery system for the concurrent administration of the model antibiotic amoxicillin trihydrate and probiotic *Lactobacillus acidophilus* (hereafter referred to as the 'Dual-Biotic System'). This system firstly underwent preformulation analyses on a denatured ovalbumin matrix to determine the physicochemical properties, stability and excipient compatibility of the proposed delivery system, after which statistical optimization of the denatured crosslinked system was undertaken. Preformulation analyses were initially conducted on an ovalbumin minitablet that was thereafter processed into granules. A correlation between the 2 systems was achieved. The statistically optimized system was then incorporated into the delayed release system, which was then included along with amoxicillin trihydrate in the Dual-Biotic System. Extensive *in vitro* characterization has been performed on the various systems formulated with both *ex vivo* and *in vivo* analyses, utilizing a Large White pig model, conducted on the final Dual-Biotic System.

1.1.3 An ideal probiotic product

Prior to the development of any probiotic delivery system, it is necessary to consider the requirements for an ideal probiotic product. Probiotics, by definition should adhere to the intestinal mucosal wall, not promote or encourage antibiotic resistance, not be pathogenic in nature and must be able to co-aggregate as part of the natural gut flora (Beachey, 1981; Iannitti and Palmieri, 2010). The degree at which functional benefits are obtained from probiotics are dependent on the type of bacteria as well as the number of viable bacteria delivered to the gastrointestinal tract (Vasiljevic and Shah, 2008; Rivera-Espinoza and Gallardo-Navarro, 2010).

There have been many studies undertaken involving the delivery of probiotics to the human gastrointestinal tract with the most recent studies focusing on targeted intestinal delivery and

protection from the harsh gastric conditions of the stomach (Chandramouli *et al*., 2004; Klayraung *et al*., 2009; Poulin *et al*., 2011). Gastric conditions have been shown to drastically decrease the number of viable probiotic bacteria with direct expose (Albertini *et al*., 2010). Various methods have been developed to deliver probiotics to the small intestine with protection from the gastric environment. These include the use of enteric coated pellets, capsules or beads, tablets using biopolymers as in addition to gastric-resistant polymers such as cellulose acetate phthalate, hydroxypropylmethylcellulose phthalate and carboxymethylcellulose high amylose starch (Chandramouli *et al*., 2004; Poulin *et al*., 2011). An ideal probiotic formulation in addition to providing gastric protection of the bacteria should furthermore also be stable against bile, oxygen and intestinal enzymes (Iannitti and Palmieri, 2010). Naturally occurring intestinal flora have an inert stability against these potentially destructive intestinal constituents, further warranting the use of natural bacteria as opposed to foreign bacterial species. The developed Dual-Biotic System was therefore designed to conform to the ideal properties of a probiotic formulation to ensure maximum therapeutic benefits to a patient. A schematic summarizing the properties of an ideal probiotic bacteria is shown in Figure 1.2.

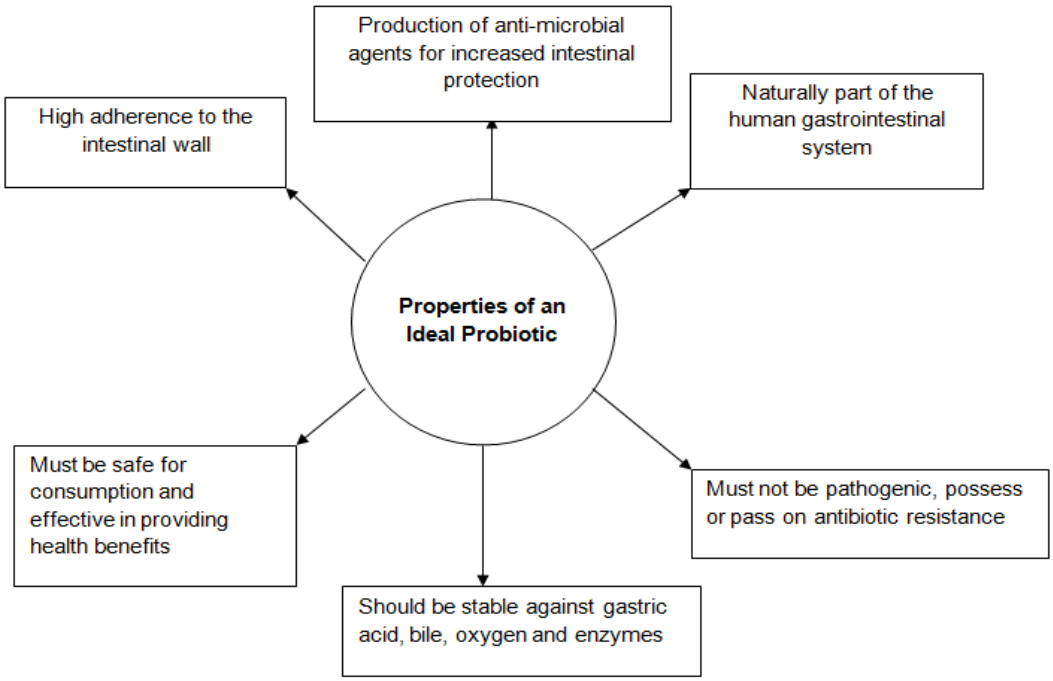


Figure 1.2: A schematic detailing the properties of an ideal probiotic bacteria (Beachey, 1981; Iannitti and Palmieri, 2010).

1.2 Rationale and Motivation for this Study

The concurrent use of antibiotics with probiotics drastically reduces the quantity of viable probiotic bacteria that attach to the intestinal or colonic mucosa (Lund *et al*., 2002). The degree of destruction of the bacteria, as described, is mainly dependent on the timing of administration of the probiotic in relation to the antibiotic formulation with the probiotic formulation ideally taken 2 hours after antibiotic administration. This is, however, not the case, with many patients ingesting the antibiotic concomitantly with the probiotic system. The formulated Dual-Biotic System is therefore proposed as a possible solution to this issue by allowing for the concurrent administration of both the antibiotic and probiotic with the probiotic release delayed to allow for the absorption of the antibiotic into systemic circulation. Amoxicillin was chosen as the model antibiotic drug due to its rapid absorption and T_{max} of approximately 1.28 hours after oral administration with variations in patients of up to 1.9 hours (Witkowski *et al*., 1982). *Lactobacillus acidophilus* has been selected as the model probiotic due to its natural occurrence in the human gastrointestinal tract and its common utilization in commercial probiotic formulations.

In order to develop a protein matrix that would be cost-effective and chemically manipulated to deliver probiotic bacteria, ovalbumin was selected. Furthermore, due to its ability to incorporate the probiotic within its matrix, it also serves to keep the probiotic bacteria in a dormant state, thereby ensuring bacterial viability over a greater period of time. A primary concern of ovalbumin however is the occurrence of egg allergies. The incidence of egg allergy, which is largely due to ovamucoid as opposed to ovalbumin, has been determined to be approximately 2% in children decreasing to 0.1% in adults (Clark *et al*., 2010). The allergy associated with ovalbumin has furthermore been shown to decrease upon change of the tertiary protein structure, mainly due to thermal denaturation (Caubet and Wang, 2011). The minimal prevalence of egg allergy, combined with the large number of individuals requiring probiotic supplementation as well as the ability of egg allergenicity to be manipulated by formulation processes, allows for ovalbumin being a viable option for probiotic delivery despite its shortcoming of egg allergy manifestations.

Current methodology for the quantification and evaluation of probiotic delivery systems is also of concern due to the microbiological analyses being, expensive, time-consuming and sometimes being inaccurate due to the large dilutions required for the visualization of colony-forming units (CFUs) (Sarker *et al*., 2007). This quantification technique is also improved on as a part of this study by evaluating a luminescence protocol to determine cell viability

through the detection of intracellular AdenoTri-Phosphate (ATP). ATP is part of the metabolic processes of bacterial cells and is not present in non-viable bacteria (Elder *et al*., 2005). The described luminescence protocol was chosen due to its accuracy and reproducibility compared to traditional *in vitro* microbiological techniques. The luminescence technique utilized is furthermore also more cost-effective, due to a single cell viability kit being adequate for hundreds of samples, as well as being time-saving due to numerous samples being run simultaneously in minutes. This improves on traditional agar plate incubation that may take a number of days to complete with the use of dilutions prior to incubation allowing for the possible introduction of experimental error.

In vivo studies of probiotic formulations are also a significant challenge with many products only scientifically, rather than clinically, proven for efficacy (more detail on the ethical and legal issues of probiotic analyses is provided in Chapter 2). Current *in vivo* analyses of probiotic formulations are also of concern as fecal matter is predominantly utilized for probiotic quantification. Due to the large number of intestinal bacteria present in the gastrointestinal tract, this methodology can allow for the inflation of results with intestinal and colonic flora contamination a distinct possibility (Marteau and Shanahan, 2003). This study also aims to improve on this methodology through the design and manufacture of a stable intestinal cannula that would allow for intestinal fluid aspiration, preventing the issue of colonic flora contamination associated with fecal matter sampling. A modified agar for the selective enumeration of *Lactobacilli* was evaluated and utilized to collaborate with the luminescence protocol and provide an accurate quantification of viable *Lactobacilli* delivered to the gastrointestinal tract.

This study therefore provides for the design and formulation of a Dual-Biotic System for the concurrent delivery of *L. acidophilus* and amoxicillin trihydrate analyzed through innovative quantification and release studies. The formulated Dual-Biotic System consists of an optimized denatured crosslinked ovalbumin system containing *L. acidophilus* encapsulated in a hydrophobic erodible system designed for a 2 hour delayed probiotic release. This delayed release system was combined with amoxicillin trihydrate in a single enteric coated hard gelatin capsule. A schematic detailing the antibiotic and probiotic release from the Dual-Biotic System is shown in Figure 1.3.

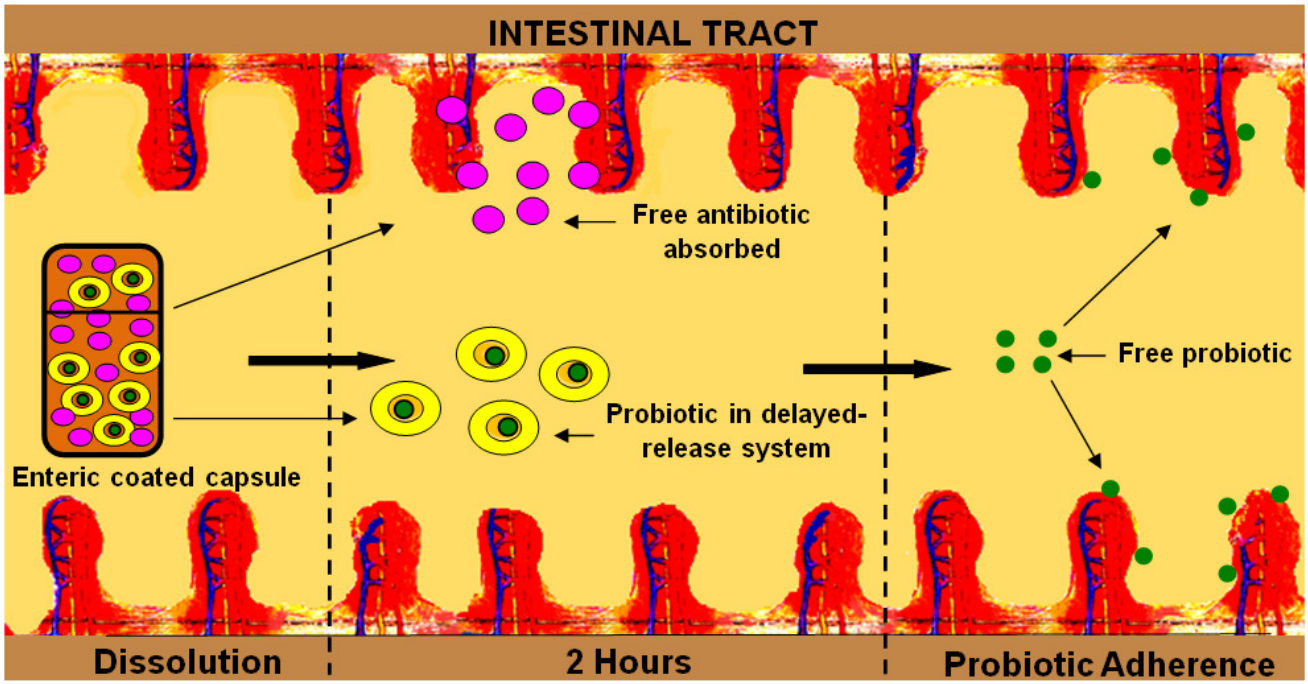


Figure 1.3: Mechanism of release and absorption from the formulated Dual-Biotic System. (Intestinal anatomy adapted from supremecalcium.com).

1.3 Aim and Objectives of this Study

The aim of this study was to formulate and evaluate an ovalbumin-based oral biopolymeric system for the site-specific concurrent delivery of amoxicillin antibiotic and *Lactobacillus acidophilus* probiotic employing a dual-release mechanism.

In order to achieve this aim, the following objectives needed to be achieved:

1. Formulation of an ovalbumin containing probiotic formulation for the protection and delivery of *L. acidophilus* to the human gastrointestinal tract.
2. Development of a luminescence protocol for the detection of viable bacterial cells.
3. Preformulation analysis of ovalbumin for the delivery of *L. acidophilus* probiotic.
4. Statistical optimization of the probiotic granule system using a Box-Behnken experimental design approach.
5. Formulation of a delayed release erodible polymer system for incorporation into the Dual-Biotic System.
6. Development of a modified *Lactobacillus*-specific agar medium for the selective enumeration of *Lactobacillus* bacteria in porcine intestinal fluid.

7. *Ex vivo* studies on excised porcine tissue to determine probiotic bacteria adhesion and probiotic protective properties such as the competitive inhibition of commonly occurring pathogenic bacteria.
8. Development of a stable cannula system for the aspiration of intestinal fluid in a pig model to allow for the quantification of delivered *Lactobacillus acidophilus* probiotic.
9. *In vivo* evaluation of the Dual-Biotic System in the Large White pig model to determine the effectiveness of the formulation to deliver viable *Lactobacillus acidophilus* probiotic concurrently with amoxicillin trihydrate antibiotic.

1.4 Potential Therapeutic Benefits of the Dual-Biotic System

Through the direct application of the Dual-Biotic System in this study, the following therapeutic benefit can be outlined:

- The supplementation of intestinal flora, in therapeutic amounts, that has been removed due to antibiotic use for the prevention of antibiotic-associated diarrhea.

Through modification and advancement of the ovalbumin-based probiotic delivery system (formulated as part of the preformulation analysis of this study), the following therapeutic application is provided for in Appendix C of this thesis.

- The delivery of probiotic bacteria site-specifically to the small intestine and colon for the treatment of generalized gastrointestinal flora deficiency through a bi-layered minitablet-in-tablet system.

1.5 Overview of this Thesis

Chapter One provides a brief introduction to gastrointestinal flora and probiotic supplementation, with the aim and objectives of this study as well as the rationale and motivation discussed.

Chapter Two provides a detailed literature review of currently available probiotic formulations. These formulations are categorized into pharmaceutical and food-based products in addition to their respective abilities to provide formulation and biological protection. A further section in this review is provided to highlight the current factors affecting probiotic therapy with ethical and legal issues impacting the effectiveness of probiotic formulations and future recommendations discussed.

Chapter Three details the preformulation analyses of this study. As part of this chapter, the ability of raw egg and purified egg ovalbumin for the delivery of probiotic *Lactobacillus acidophilus* is evaluated in addition to the effect of protein denaturation on probiotic release. Further provided in this chapter are the effects of formulation processes on *Lactobacillus acidophilus* release as well as the mechanism of gastric protection provided by the ovalbumin matrix and subsequent release in simulated intestinal media utilizing molecular docking studies.

Chapter Four details the design and statistical optimization of the crosslinked denatured ovalbumin granule system, utilizing a Box-Benkhen experimental design approach, as the active therapeutic component of the Dual-Biotic System. Further provided in this chapter is the evaluation of the optimized ovalbumin system through comparison of predicted and observed values to predetermined design responses. Atomistic simulations as well as energetic paradigms of the crosslinked ovalbumin matrix are also provided as part of this chapter.

Chapter Five describes the *in vitro* characterization of the optimized ovalbumin granule system and details the design, formulation and analysis of the various components of the Dual-Biotic System.

Chapter Six outlines a modified MRS agar that has been developed for the selective enumeration of *Lactobacillus* bacteria in aspirated porcine intestinal fluid. It further describes the *ex vivo* evaluation of the formulated Dual-Biotic System. Analyses performed highlight the adhesion potential of the encapsulated probiotic bacteria and compares it to a conventional probiotic formulation evaluated in the *in vivo* component of this study. The protective ability of *L. acidophilus* against foreign pathogenic bacteria is also analyzed and discussed.

Chapter Seven describes the *in vivo* evaluation of the Dual-Biotic System in the Large White pig model. Further described is the design and manufacture of a stable intestinal cannula that was surgically implanted into the intestinal ileum of the pig for the aspiration of intestinal fluid and determination of *Lactobacillus acidophilus* cell counts. Results are compared with conventional antibiotic and probiotic formulations analyzed as part of the *in vivo* study. Pharmacokinetic modeling analysis is also described in detail with the development of an *in vitro-in vivo* correlation.

Chapter Eight outlines the conclusion of this study and details future recommendations for advancing of the Dual-Biotic System. Modifications of the manufactured intestinal cannula for prolonged usage are also provided as part of this chapter.

CHAPTER 2

ADVANCEMENTS IN PROBIOTIC DELIVERY: PHARMACEUTICAL FORMULATIONS VERSUS FOOD-BASED PRODUCTS FOR INTESTINAL FLORA SUPPLEMENTATION

2.1 Introduction

The use of intestinal flora supplementation can be dated back decades with the first documented article on probiotics published in the early 20th century by Elie Metchnikoff. Probiotic research has, however, greatly increased in the last decade with over 5000 publications detailing their health benefits as well as the ability to delivery viable functional probiotic bacteria (Verna and Lucak, 2010). The main species of bacteria used in probiotic formulations are *Lactobacillus* and *Bifidobacterium* spp, which are classified as anaerobic bacteria and therefore require an oxygen-free environment for growth to occur (Alander *et al*., 2001; Penner *et al*., 2005; Dworkin *et al*., 2006; Spinler *et al*., 2008; Quigley, 2010; Cook *et al*., 2012). Potentially advantageous properties of probiotic bacteria such as gastric-resistance vary from species to species and within strains. For example, *Lactobacillus* spp. are more viable in gastric conditions compared to other probiotic species, making it the most ideal probiotic (active ingredient) for dosage forms that do not provide gastro-protection (Maragkoudakis *et al*., 2006). Some species of bacteria are also found in greater numbers in certain parts of the gastrointestinal tract and provide a more functional role when they colonize these areas. An example of this is *Bifidobacterium* which is found in large numbers in the colon and serves as a digestion aid to ferment and digest complex carbohydrates from the host's diet (Rabiu and Gibson, 2002). The advantage of this property is that it allows for targeted, species-specific probiotic delivery systems which will result in greater health benefits to the patient.

2.1.1 Probiotic delivery mechanisms

Many systems have been developed for the delivery of probiotics to the gastrointestinal tract and include both pharmaceutical and commercially available formulations. These commercial formulations consist mainly of food-based products, many of which use probiotic bacteria in their production, with others having added these bacteria as an adjunctive health benefit of product ingestion. These products account for 90% of probiotic formulations and with the large amount of research into the improvement of commercially available food-based products for the delivery of functional probiotic bacteria, their ability to act as probiotic

delivery systems cannot be ignored. Food-based probiotic formulations range from cheeses, yogurts, creams, chocolates, milk and meat, amongst others. They have been around for decades and are sold to the general public with little or no regulation and control (Stanton *et al*., 1998; Joosten *et al*., 2006; Khan *et al*., 2011; Cousin *et al*., 2012). Due to their easy availability and convenience, these products are good delivery systems that, if effective, can be highly beneficial to the patient. A few of these products have the ability to deliver viable probiotic bacterial cells to the human intestine but as with pharmaceutical formulations, differ greatly. This variation is as a result of various reasons ranging from formulation processes, viability of the dosed bacteria, as well as the ability of different species of bacteria to survive physiological conditions and adhere to the intestinal wall.

Pharmaceutical products tend to be more effective in this regard and are much more characterized compared to the commercial food-based carrier systems. Examples of pharmaceutical formulations for the delivery of probiotics currently include, amongst others, beads, capsules and tablets (Schrezenmeir and de Vrese, 2001; Cook *et al*., 2012). Whilst each type of formulation has been found to possess advantages in the delivery of probiotics, with each delivering varying amounts of viable probiotic bacterial cells, differences in their effectiveness to deliver the correct amount of bacteria as well providing protection to the dosed probiotic bacteria exists. Formulation processes also seem to affect the ability of dosage forms to deliver the required numbers of viable bacteria with processes, such as lyophilization, affecting the stability of probiotic bacteria. With these issues, various pharmaceutical changes have been incorporated into formulations to not only improve probiotic bacteria survival during formulation processes, but in both *in vitro* and *in vivo* studies seek to improve on the issues of physiological viability of probiotic bacteria.

Challenges do, however, exist in both categories of probiotic delivery systems and have been thoroughly researched. Many newer systems seek to alleviate these issues and provide effective functional delivery systems that will provide health benefits to the patient. The challenges that exist in delivery of probiotics in both the pharmaceutical and commercial products include lack of protection in the harsh gastric environment, delivery of inadequate amounts of viable bacteria at the time of administration, delivery of the incorrect strains of probiotic bacteria, as well as little protection against the concurrent delivery of antibiotics (Lund *et al*., 2002; Mattila-Sandholm *et al*., 2002; Ding and Shah, 2007; Siepmann *et al*., 2008). Studies have shown that at least 10^8-10^9 viable cells must reach the intestine for health benefits to be achieved for the patient (Hou *et al*., 2003; Doleyres and Lacroix, 2005; Kailasapathy, 2006; Oliveira *et al*., 2009). Many formulations tend to deliver species of

probiotic bacteria that have more gastro-resistant properties. *Lactobacillus* spp tend to have a greater resistance to gastric acid than others species such as *Bifidobacterium* and are found more often in probiotic formulations (Krasaekoopt *et al*., 2004; Iannitti and Palmieri, 2010; Jensen *et al*., 2012). *Enterococci* have also been found to be more resistant to gastric conditions compared to other bacteria, with 66% of tested bacteria surviving 60 minutes at pH 3.0 and 40% in pH 2.0. This was considered acceptable in the delivery of bacteria to the stomach as the average human stomach is between pH 2.0 and 3.0 (Bhardwaj *et al*., 2010). Bile tolerance is also an important property required in probiotic bacteria due to the interaction of the bacteria with bile on entry into the small intestine. This issue, however, is not seen with naturally occurring probiotic bacteria as they have developed tolerance being commonly exposed to bile salts in the intestinal system (Bhardwaj *et al*., 2010).

This chapter will therefore provide a brief description of the health benefits of probiotic supplementation, elaborate on the different ways in which probiotic formulations are prepared, distinguish between the differences between pharmaceutical and food-based products for the delivery of probiotic bacteria and will further discuss the current issues facing probiotic delivery. In addition newer analyses that are currently being used for the accurate determination of probiotic delivery system effectiveness will be given attention, as well as recommendations made for future advancements in probiotic delivery. A brief introduction to how the formulated Dual-Biotic System of this study overcomes current factors affecting probiotic therapy is also provided.

2.1.2 Therapeutic advantages of probiotic supplementation

Probiotic bacteria are currently classified as nutraceuticals and account for billions in sales annually (Cook *et al*., 2012). Nutraceuticals are defined as dietary substances that deliver a concentrated form of a bioactive substance in quantities that exceed what can be obtained from food (Penner *et al*., 2005). Intestinal flora supplementation through the use of probiotics has been shown through many studies to be effective in the prevention and treatment of conditions caused by pathogenic bacteria. This treatment has been shown to occur due to the probiotic's competitive inhibition of pathogenic bacteria through the adhesion and colonization of binding sites in the small intestine (Collado *et al*., 2007; Copeland *et al*., 2009). It is this preventive role, amongst others, of intestinal flora that's of vital importance for the health and overall well-being of humans. Probiotic bacteria have, however, been shown to exert health benefits over and above simple adhesion inhibition and aid in digestion. These health benefits extend into immuno-modulation, cancer therapy and cholesterol lowering.

2.1.2.1 *Immuno-stimulatory responses and biological activities of probiotics*

In addition to the prevention of adhesion and colonization of pathogenic bacteria, probiotic bacteria have been found to provide immuno-stimulatory responses. Whilst the positive effects of the oral ingestion of bacteria have been widely debated, more health benefits have been shown to exist above gut-specific functions and immunological functions leading to the belief that probiotics have functional benefits at both a cellular and molecular level (Panigrahi *et al*., 2007). The proposed method of treatment by probiotics is that the human gastrointestinal system routinely samples gut microflora to assert with its regulatory functions and that probiotic supplementation assists in this process (Panigrahi *et al*., 2007). A study into the effects of probiotics on fish microflora has shown that probiotic bacteria assist in the expression of cytokines and other inflammatory mediators. Further analysis of the immunological effects of probiotic bacteria showed an increase in survival rates with animals exposed to *Vibrio alginolyticus* (Tseng *et al*., 2009). The test organism (shrimp) in the study showed increases in phenoloxidase activity, phagocytic activity and clearance efficiency of the inoculated pathogen compared to shrimp that were not fed a steady probiotic diet (Tseng *et al*., 2009). This increase in survival was, however, proportional to the number of bacteria delivered to the shrimp (Tseng *et al*., 2009). It was therefore hypothesized that probiotic bacteria have immunological effects far beyond protection from colonization and adhesion, but may also assist in both the prevention and eradication of pathogenic bacteria within the test organism.

With the intestinal mucosa exposed to a variety of pathogenic bacteria, vaccines are a crucial intervention that is currently being sought for the prevention of many life-threatening conditions. Probiotic bacteria, which are organisms that are naturally part of the intestinal mucosa, have the potential to be a delivery system of vaccines for the prevention of conditions caused by pathogenic organisms in the intestine. Due to the invasive nature of current vaccine formulations, research into the use of orally induced live carriers capable of expressing specific pathogenic virulence factor-derived antigens to exert an immunological response has occurred. *E. coli* Nissle 1917 is an example of such a probiotic bacterium that has been linked to a possible use as a vaccine delivery system (Buddenborg *et al*., 2008). This nonpathogenic bacterium, when utilized as a live carrier, has been shown in this study to successfully exert an immunological response in rats when coupled with model antigens. This immunological response, determined through specific antibiotic titers, was, however, not consistent and determined to be sub-effective. Due to the results seen in this study, the use of probiotic bacteria in vaccine development further highlights the possibility of utilizing probiotic bacteria in immunological modulation. This study furthermore highlights a highly

promising area of probiotic research still to be fully undertaken, with the possibility of groundbreaking discoveries to be found.

Lactobacillus bacteria have also been shown to have anti-allergy properties in the prevention of atopic sensitization (Cross, 2002; Sunada *et al*., 2008). The proposed method for this immune-modulation is that *Lactobacillus*, as well as other probiotics, potentiates T-helper [Th] cells influencing the formation of pro-Th cytokines. This immune-modulation has, however, been shown to be strain-specific and not applicable to all strains of *Lactobacillus* bacteria (Cross, 2002). Images of mice predisposed to atopic dermatitis treated with *L. acidophilus* strain L-55 as well as with prednisolone, a common treatment for atopic dermatitis are depicted in Figure 2.1.

Probiotic bacteria have also been linked to cancer therapy. A wide variety of studies have been conducted to determine the effectiveness of systemic administration of both pathogenic and non-pathogenic bacteria for the possible site-specific protein treatment of cancer patients bearing tumors. The mechanism of delivery of drugs using bacteria is that bacteria agglomerate within cancer tumors but significantly less in organs such as the spleen and liver resulting in less toxicity expected with the systemic administration of bacteria (Stritzker *et al*., 2007). The use of probiotic bacteria, which by definition are non-pathogenic and would have significantly less systemic side-effects, has been researched with *E. coli* Nissle 1917. Results of this study showed a large number of bacterial cells agglomerating in tumors within mice, proposing probiotic bacteria as a possible tumor-targeting mechanism that can be used in both immuno-competent and immuno-compromised patients, which in the treatment of cancer patients is vital (Stritzker *et al*., 2007). Probiotic bacteria have been further found to be therapeutic in patients suffering from high cholesterol levels as well as in patients diagnosed with obesity (Grzeskowiak *et al*., 2012; Makinen *et al*., 2012). A study conducted by Guerra *et al*. (2007) has also shown that probiotic bacteria can also be used as an alternative growth enhancer in pigs, a commonly used *in vivo* test model for correlation with human patients.

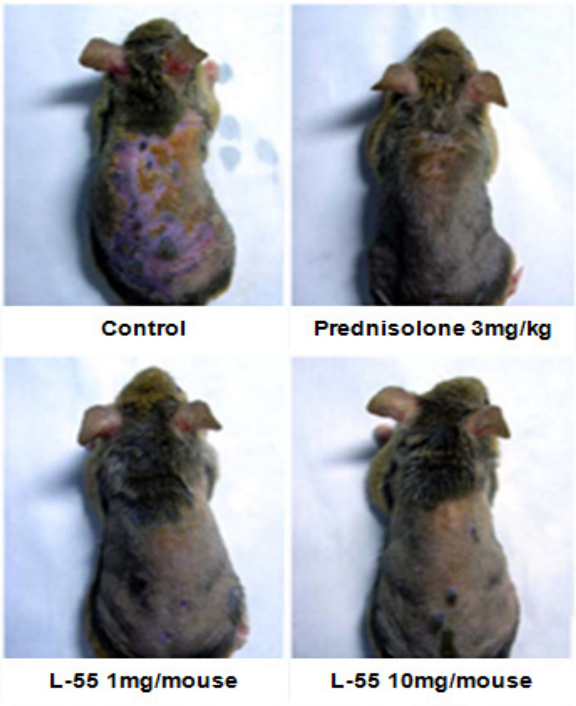


Figure 2.1: Images of mice predisposed to atopic dermatitis treated with prednisolone and *L. acidophilus* strain L-55 showing significant improvement in the treatment of this condition using the probiotic bacteria when compared to the control group. Image reproduced from Sunada *et al*. (2008).

2.1.2.2 *Concomitant antibiotic use and probiotics*

Antibiotics have been proven to disturb intestinal flora balance by killing susceptible intestinal microbes. Probiotic supplementation traditionally seeks to increase the numbers of intestinal flora after antibiotic administration, preventing commonly seen side effects of antibiotic therapy. A study conducted by Madden *et al*. (2005) have indicated that in patients receiving multiple forms of antibiotics, probiotics are effective in restoring intestinal flora removed by the antibiotic therapy. Commonly used probiotic bacteria (*L. acidophilus* and *Bifidobacterium bifidum)* showed an increase in colony numbers after the therapeutic treatment of antibiotics and indicated that the possibility of the use of these supplementary bacteria are vital in preventing any permanent changes in intestinal flora after antibiotic treatment. This study also showed that the susceptibility of intestinal flora varies from species to species, with potentially pathogenic bacteria, such as those found in the *Enterobacteriaceae* family and *Staphylococci*, increasing in numbers after 14 days compared to other species of naturally occurring intestinal flora. The ideal probiotic formulation may therefore need to be resistant to the effects of the administered antibiotic so that it may protect the patient from potentially pathogenic bacteria such as *Staphylococci* but may not, by definition, pass on this resistance to other bacteria which may potentially have

life-threatening consequences. With this issue of antibiotics removing functional bacteria that protect the human body, it has been shown that probiotic bacteria may be a substitute for antibiotics for certain gastrointestinal conditions. Furthermore, with the ability of probiotic bacteria to compete for adhesion sites, species of pathogenic bacteria have been shown to decrease upon administration of probiotics with up to a 40% decrease in pathogenic bacteria dependent on the species of bacteria causing the infection (Collado *et al.*, 2007). *Lactobacillus* has been shown to produce an antimicrobial agent that is effective against a variety of potentially pathogenic bacterial species, such as *Clostridium*, *Bacteroides*, *Pseudomonas*, *Staphylococcus* and *Streptococcus*, which commonly infect the intestinal tract. The produced antimicrobial agent, being effective against a relatively wide spectrum of bacteria did not, however, affect the viability of *Lactobacillus* bacteria (Silva *et al.*, 1987).

2.1.2.3 *Other bacterial species that provide health benefits*

Bacterial species not strictly classified as probiotics have also been shown to provide health benefits to patients. One such example is *Enterococcus faecium*, which have been shown to provide functional benefits in the treatment of *Salmonella* infections (Bhardwaj *et al.*, 2010). A comparison on the functions of the various species of probiotic bacteria and some of their physiological functions are listed in Table 2.1 with a brief summarization on the physiological functions of intestinal flora represented in Table 2.2.

Table 2.1: Comparative analysis of probiotic bacteria genera and their functional health benefits.

Genus (Probiotic spp. included)	Functional benefit (Of Genus)	Reference
Lactobacillus	Prevention of vaginosis	Santiago *et al*. (2009)
L. acidophilus	Antibiotic-associated diarrhea	Douglas and Sanders (2008)
L. fermentum	Infant diarrhea	Douglas and Sanders (2008)
L. helveticus	Atopic dermatitis	Douglas and Sanders (2008)
L. paracasei	Promotion of vitamin production	Mombelli and Gismondo (2000)
L. rhamnosus	Digestion	Mombelli and Gismondo (2000)
L. salivarius		
Bifidobacterium	Irritable bowel disease	Douglas and Sanders (2008)
B. bifidum	Gut transit time control	Douglas and Sanders (2008)
B. breve	Immune support	Douglas and Sanders (2008)
B. longum	Antimutagens	Mombelli and Gismondo (2000)
	Anticholesterol agents	Mombelli and Gismondo (2000)
	Digestion	Mombelli and Gismondo (2000)
Enterococci		
E. faecium	Treatment of gastroenteritis and *Salmonella* infections	Bhardwaj *et al*. (2010)
Escherichia	Anti-tumor	Stritzker *et al*. (2007)
E. coli Nissle 1917	Vaccine delivery	Buddenborg *et al*. (2008)

Table 2.2: Biological health effects of functional probiotic bacteria. Adapted from Scheinbach (1998) and Penner *et al*. (2005).

Biological Health Effects			
Stimulation of the immune system	**Epithelial barrier protection**	**Anti-microbial effects**	**Other health benefits**
Enhanced antibody production	Enhanced tight junction protein phosphorylation	Production of acids, peroxides or bacteriocins bactericidal to groups that negatively impact health	Alleviation of lactose intolerance Cholesterol reduction
Enhanced natural killer cell activity	Upregulation of mucous production	Stimulation of defensin secretion	Tumor targeting
Modulation of dendritic cell phenotype and function	Enhanced epithelial cell glycosylation	Secretion of anti-microbial peptides	
Modulation of NF-kB and AP-1 pathway	Increased sIgA production	Inhibition of pathogenic bacterial invasion	
Altered cytokine release	Competition with pathogens for mucosal binding sites	Blockade of bacterial adhesion to epithelial cells	
Induction of regulatory T- cells	Competition for substrates	Release of nitric oxide	
Induction of PPAR-g			
Modulation of apoptosis			
Inhibition of proteasome activity			

2.2 The Ability of Pharmaceutical and Food-Based Formulations to Deliver Therapeutic Doses of Probiotics

2.2.1 Delivery of viable functional probiotic bacteria

With the large number of probiotic formulations available, a large variation in the viability and thus the effectiveness of the delivery systems exists. Due to the need to deliver probiotic bacteria to the small intestine and colon, the most common and effective delivery route is orally, with the most chosen delivery systems being tablets or capsules, both with or without chemical and structural modification. Tablets have been shown to be good delivery systems for the delivery of probiotics with the manipulation of tablet excipients drastically improving the number of viable cells reaching the human intestine. With the addition of gastro-resistant polymers, adhesion enhancers and controlled release enhancers, the manipulation of formulation excipients have overcome many challenges facing effective probiotic delivery. Furthermore, with tablets and capsules being easier to administer to patients and due to their increased storage stability over liquid preparations, capsules and tablets are currently the preferred dosage forms for the delivery of probiotics to the human body (Dash *et al*., 1999). The concern with tablets, however, is the heat production that occurs during

compression with temperatures reaching up to 60℃. This can result in the destruction of bacterial cells which are most viable at their optimal temperatures (Roueche *et al*., 2006; Panigrahi *et al*., 2007). Other pharmaceutical delivery systems for probiotics include microcapsules and beads, which have been shown to drastically increase the number of viable bacteria surviving the formulation process, storage period and subsequent delivery to the intestinal tract (Krasaekoopt *et al*., 2004; Cook *et al*., 2012). The degree of bacterial survival in these formulations was, however, dependent on the polymer combinations used, the formulation processes undertaken and the size of particulates formed. The survivability of the probiotic bacteria, however, was ultimately shown to be substantially more in these formulations when compared to the unmodified probiotic bacteria.

With commercial food products, cheeses have been commonly used as carriers for probiotic delivery. Ong *et al*. (2006) showed the ability of three batches of cheddar cheese to deliver a variety of probiotic bacteria. Results showed that the Cheddar cheeses can be an effective vehicle for delivery of the *Lactobacillus* and *Bifidobacterium* strains tested. Fresh cheese was also found to be a suitable vehicle for the oral administration of *B. bifidum*, *L. acidophilus Lactococcus lactis*, *Lactobacillus paracasei* and *Streptococcus thermophilus* (Medici *et al*., 2004). A study conducted on the ability of cheese to deliver *L. acidophilus* and *L.paracasei* was done by influencing the stage during the cheese making process at which the probiotic was added. All tests conducted showed an increase of intestinal flora after ingestion of the probiotic cheese (Minelli *et al*., 2004). Similar studies were conducted by Phillips *et al*. (2006) to deliver probiotics through cheddar cheese with results showing significant increases in all species tested except for *L. acidophilus*. Bergamini *et al*. (2005) and Ong and Shah (2009) also showed the ability of Argentinean and cheddar cheese respectively to deliver probiotic bacteria to the human GIT.

Milk has also been documented for the delivery of probiotics. The delivery of *Lactobacillus* species showed an increase in colony numbers after administration through milk (Minelli *et al*., 2004). The use of milk was further studied by the possible use of Caseinomacropeptide in milk to deliver *Bifidobacterium lactis* (Janer *et al*., 2004). Caseinomacropeptide is a glycopeptide that contains nitrogen and amino-sugars that act as a growth substrate for *Bifidobacteria*. Results showed an increase in microbial growth with a variation dependent on the concentration of caseinomacropeptide used. The use of yogurts in the delivery of probiotics has also been widely documented (Lourens-Hattingh and Viljoen, 2001). Hemsworth *et al*. (2011) showed the ability of yogurts to deliver probiotics. *Lactobacillus rhamnosus* cell counts increased after administration of the yogurt which included the

probiotic, milk and macronutrients. A study conducted by Marafon *et al*. (2011) showed the ability to optimize the use of yogurts as delivery systems for probiotics. In the study, a starter culture blend that consisted of *B. lactis*, *Lactobacillus bulgaricus*, and *S. thermophilus* was used. Results showed that the addition of milk proteins of *Lactobacillus bulgaricus* only decreased significantly leaving the possibility of optimization of the other strains of bacteria. A review conducted by Lourens-Hattingh and Viljoen (2001) on the use of yogurts to deliver probiotics showed that with an increase in the use of yogurts to supplement intestinal flora by the general public, a deeper investigation into the effectiveness of these yogurts is required. The ability of yogurts to deliver probiotic bacteria was also described to be low in comparison to other food sources due to factors such as acidity and oxygen content which do not favor the growth of naturally occurring bacteria such as anaerobic *Bifidobacterium*.

The use of other food products has also been identified for the delivery of probiotics. A study conducted by Possemiers *et al*. (2010) showed the possibility of chocolate coatings for the protection and delivery of *Bifidobacterium longum* and *Lactobacillus helveticus*. Results showed significant survival of bacterial cells leading to the belief that chocolate can be used to protect and deliver probiotics as well as other gastro-sensitive products (Possemiers *et al*., 2010). The use of other chocolate-based products such as chocolate mousse has also been utilized to deliver probiotics (Aragon-Alegro *et al*., 2007). Chocolate mousse supplemented with *L. paracasei* determined a high degree of survival of the bacteria upon administration (Aragon-Alegro *et al*., 2007). The use of ice cream in the delivery of probiotics has also been explored with ice creams showing greater protection of bacteria in simulated gastric conditions compared to milk and yogurts (Senaka Ranadheera *et al*., 2012). This was hypothesized to be due to the higher fat content in ice cream, (approximately 10%), which may have provided a greater degree of protection against gastric acid and bile salts compared to the yogurt which had an approximate fat content of 5%. The ingredients in the ice cream, such as cocoa powder and stabilizers were also believed to provide protection against the harmful effects of the gastric acid and bile. This further explains the protective effects of chocolate-based products, as cocoa is a significant part of chocolate itself. Probiotics have also been delivered through maple sap which was supplemented with *B. lactis* and *L. rhamnosus* with and without inulin (Aragon-Alegro *et al*., 2007; Khalf *et al*., 2010). The inulin significantly enhanced the survival of the bacteria in simulated gastric and intestinal conditions (Khalf *et al*., 2010).

2.2.2 Freeze dried formulations and advancements with cryo-protection

Lyophilized probiotic bacteria have been widely used as the chosen form of probiotic bacterial delivery to the human gastrointestinal tract. The reason for this trend is that lyophilization of these bacterial cells preserves their viability, due to their low water activity, as well as improving the stability and viability of the formulation itself (Kos *et al*., 2008). Freeze-drying also been shown to not have an effect on the ability of probiotic bacteria to protect the intestinal system from pathogenic bacteria, with a test pathogen of *Shigella sonnei* co-incubated with probiotic bacteria after lyophilization showing a significant decrease in *Shigella sonnei* levels after the test period (Bolla *et al*., 2011). Lyophilization on its own, however, has been shown to be detrimental to the survival of bacteria and therefore protection against the harsh effects of freeze-drying is most often needed (Zarate and Nader-Macias, 2006). Cryo-protection seems to be the answer to the issue of freeze-drying on cell viability. This leads to a better treatment regimen due to greater numbers of viable bacteria reaching the intestinal system, as well as a decrease in the cost of manufacture as a result of the decreased loss in functional bacteria using relatively cheap cryo-protectants. The type of cryo-protectant used, however, also determines the degree of viability during lyophilization as well as during shelf-life and storage. Traditionally milk based products such as lactose and skimmed milk have been used as cryo-protectants for the protection of bacteria during lyophilization but this protection is short-lived during the shelf-life of the product, with bacteria viability decreasing only after a few months of storage (Zarate and Nader-Macias, 2006; Vincenzetti *et al*., 2011). Other excipients such as ascorbic acid, a mild antioxidant, have been shown to be more effective in the protection of viability of bacteria during shelf-life with a possible combination of milk and ascorbic acid in lyophilization of probiotic bacteria being the most effective (Zarate and Nader-Macias, 2006). A study conducted by Savini *et al*. (2010) further showed the cryo-protective properties of polyalcohols, glycerin, sorbitol, mannitol and prebiotic oligosaccharides inulin, Crystalean® starch and dextrin. Results of this analysis determined that all the tested cryo-protectants were effective in the protection of probiotic bacteria during freeze-drying with little change seen in bacterial cell counts when the bacteria were stored at 4℃ for 5 months. A difference, however, was seen when the cultured probiotic were exposed to room temperature for the tested time period. A significant decrease in bacterial cell counts was seen over time with glycerin showing the best protective properties during storage as compared to other compounds tested. This was determined to be attributed to the penetrating properties of glycerin over the tested oligosaccharides and being more active than the other polyalcohols analyzed.

An issue seen with many freeze-dried probiotic formulations however, is when they are added to liquid preparations prior to storage (seen in many yogurts and other food-based products), the result is that ultimately rehydrating the freeze-dried bacteria decreases the stability of the probiotics, affecting their viability through storage (Heidebach *et al*., 2010). This issue was investigated by Weinbreck *et al*. (2010) which showed that dried encapsulated *Lactobacillus* bacteria when exposed to water over a two week period significantly decreased the viability of the encapsulated bacteria, further proving that even when encapsulated, bacteria will have to remain dry to the point of delivery to have the viability needed to exert the required health benefits. This decrease was further explained by Vesterlund *et al*. (2012) who showed that when dried foods containing probiotic bacteria was exposed to or contained water, the viability of probiotic bacteria during the shelf-life of the product decreased considerably. Over-drying of probiotic bacteria, however, can be detrimental to bacterial survival rate over time (Zayed and Roos, 2004). This was due to the biological nature of bacterial cells, where a 0.0% moisture content revealed a bacterial viability decrease of 44% within one week of storage when compared to bacteria containing a moisture content of 2.8%. It was therefore determined that an ideal moisture content for the probiotic bacteria analyzed, *Lactobacillus salivarius*, was between 2.8 to 5.6%, where a moisture content of 8.8% and over resulted in a large decrease in bacterial viability over time. These values were, however, specific to the bacteria tested and would vary from species to species.

An alternative to cryo-protection is the use of microencapsulation, which is also used to protect probiotic bacteria during freeze-drying. Microencapsulation using polysaccharide or protein based systems has been shown to be far more effective in the protection of bacteria during freeze-drying and storage as compared to traditional cryo-protection. Combined with the effect of polysaccharides, which are used as prebiotics, this allows for a suitable delivery system that protects the delivered probiotic bacteria and has the added effect of producing a synbiotic formulation (Heidebach *et al*., 2010). Prebiotics by definition, provide growth enhancers and nutrients that assist in the growth of probiotic bacteria when delivered to the small intestine. Synbiotics are defined as a “combination of pre- and probiotics”. The most commonly used prebiotics in Europe are fructo-oligosaccharides [FOS], which are naturally found in a variety of vegetables such as asparagus, leeks, artichokes, onions and garlic (Vitali *et al*., 2012).

The parameters of the freeze-drying process have also been shown to have a large effect on bacterial viability. This effect has been shown to be strain specific with certain species of

probiotic bacteria being capable of surviving lower temperatures when compared to other bacterial species. An example of a bacterium that is unstable at low temperatures is *Lactobacillus delbrueckii*, a probiotic whose numbers decrease drastically at temperatures below 0℃ (Bauer *et al*., 2012). In comparison, *L. paracasei*, has been shown to survive at much lower temperatures, commonly associated with freeze-drying, with a significantly larger proportion of bacterial cells surviving the formulation process. This difference was shown to be attributed to the membrane structure of the respective bacterial cells affecting the resistance of the bacteria against low temperatures. Low temperature vacuum drying (LTVD) is therefore proposed as an alternative to freeze-drying due to the lower temperature ranges utilized and higher viable bacteria yields seen in cryo-labile bacteria such as *L. delbrueckii*.

Storage conditions of probiotics before and after formulation processes are also an important factor in the viability of the delivered probiotic bacteria. Probiotics have been shown to survive in greater numbers when stored at -70℃ prior to the formulation process compared to when stored at 7℃ in a refrigerator. This was due to the cryo-protectants used such as glycerol, milk etc that prevented intracellular formation of ice within the bacteria, thus preventing a decrease in their viability when frozen (Juarez Tomas *et al*., 2004). The issue that arises, however, from storing probiotic bacteria at frozen temperatures is the problem of transportation and cold storage across great distances. This can be solved by transportation of cultures to the site of culturing and processing, and maintaining a cold chain from production to the patient (Juarez Tomas *et al*., 2004). It has also been shown that the presence of other bacteria in the formulation, oxygen content, the amount of acid-producing bacteria as well as the temperature affected the viability of probiotic bacteria in liquid or semi-solid products such as yogurts (Dave and Shah, 1997).

2.2.3 Protection of probiotics against gastric conditions

A major issue in probiotic therapy is the ability of formulations to protect probiotic bacteria from the harsh gastric environment, with as much as 60-100% of probiotic bacteria being killed in the gastric environment prior to reaching the intestine where the bacteria will exert their health benefits (Bezkorovainy, 2001). This number depends on the species of bacteria delivered with some species showing more protection in the gastric environment compared to others. Gastro-resistant polymers and coatings have been shown to supply gastric protection. These coatings included enteric-coated tablets and capsules that site-specifically deliver the administered probiotic bacteria to the intestinal system. These enteric coats are often pH-selective and allow for the protection against the gastric conditions and

subsequently dissolve in the alkali media of the intestinal tract. Hydroxypropyl methylcellulose phthalate has been used to deliver *Lactobacillus fermentum* under simulated human gastric and intestinal conditions (Klayraung *et al*., 2009). It was found that the hydroxypropyl methylcellulose phthalate not only protected the probiotic bacteria but also contributed to the development of hard tablets, with a high tensile strength, with bacterial viability still intact (Klayraung *et al*., 2009). Other excipients that have gastro-resistant properties and have been used for probiotic delivery is carboxymethyl high amylose starch. Starch is a commonly used filler and binder in tablet manufacturing and is polysaccharide in nature. However, when chemically substituted, it yields a starch derivative capable of protecting bacteria in, the gastric environment. It has been used previously to deliver *E. coli* as a vaccine against neonatal and post-weaning diarrhea in pigs (Calinescu *et al*., 2005). Being polysaccharide in nature, the high amylose starch was quickly dissolved by enzymatic hydrolysis upon reaching the small intestine and safely delivered the *E. coli* bacteria into the intestine for an immunological response to take place. Tests done on the formulation showed a high survival of viable bacterial cells reaching the small intestine after being exposed to gastric acid leading to carboxymethyl high amylose starch being an effective carrier for gastric-sensitive products for intestinal delivery.

This concept was also again seen in the use of carboxymethyl high amylose starch for the delivery of *L. rhamnosus* to the colon. Colon drug delivery requires that the triggering mechanism of the delivery system has to only respond to the physiological conditions particular to the colon in order to prevent release in the stomach or intestine (Yang *et al*., 2002). The carboxymethyl high amylose starch was combined with chitosan for delivery of the probiotic to the colon. The resultant chitosan-carboxymethyl high amylose starch combination swelled upon reaching the small intestine, due to the hydrating properties of chitosan, and thereafter released the probiotic into the colon. It therefore became possible to site specifically deliver probiotics to the human intestine and colon. Other studies also confirm that different polymers with different properties, or similar polymers with different properties, can be used, depending on the site of intended delivery to deliver an active ingredient, in this case probiotic, to different areas of the gastrointestinal tract (GIT) (Yang *et al*., 2002). The effectiveness of carboxymethyl high amylose starch has also been documented to deliver F4 fimbriae, another gastro-sensitive product to the intestinal tract (Calinescu *et al*., 2007). A schematic derived from Yang *et al*. (2002) is shown in Figure 2.2 and shows the mechanism of release of a commercial colon-targeting dosage form as it passes through the gastrointestinal tract in which colon microflora is utilized for site-specific drug delivery.

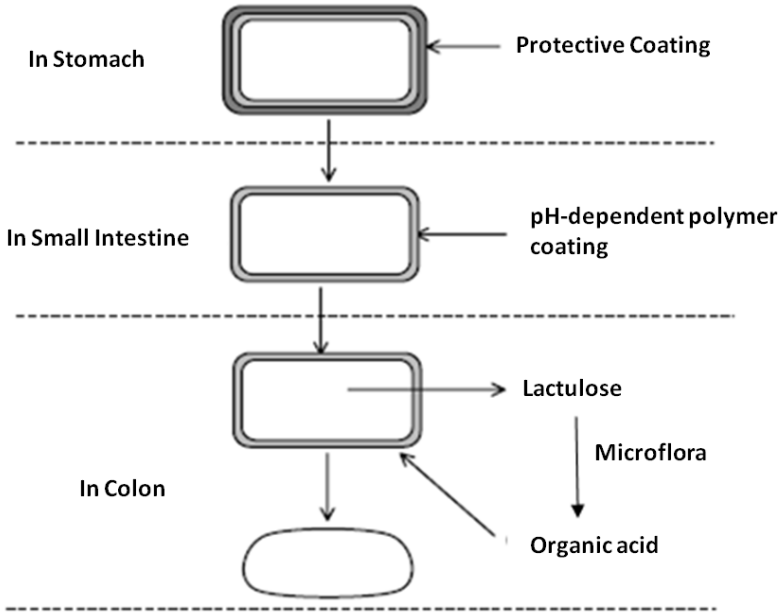


Figure 2.2: A schematic depicting the use of colon microflora for site-specific drug delivery. Adapted from Yang *et al*. (2002).

Compression coatings have also been effective in the protection of probiotic bacteria from gastric conditions. A compression of sodium alginate, a gelling agent that erodes over time in the GIT has been used for the protection of probiotic bacteria from gastric conditions. Results of a study conducted by Chan and Zhang (2005) showed a significant increase in the survival rates of bacteria when exposed to gastric conditions with the resultant dosage form able to deliver the probiotic bacteria late in the small intestine and in the early colon. More recent studies have been attempting to deliver probiotics through the use of biopolymers such as succinylated β-lactoglobulin, a protein commonly found in dairy products. This β-lactoglobulin protein was found to possess gastro-resistant properties once modified and through a compressed tablet, was able to deliver viable *B. longum* bacterial cells to the intestine (Poulin *et al*., 2011). Commercial food-based products such as proteins and carbohydrates have also been proven to be effective in the gastric protection of probiotic bacteria. Whey protein isolate, a by-product of cheese manufacture and commonly found in other food products has been shown to be effective in the protection of *Lactobacillus* bacteria in simulated gastric conditions. The whey protein isolate beads also tended to be more stable as compared to alginate beads which have a limited stability once formulated (Doherty *et al*., 2011). Further studies into the use of commercial food based products for encapsulation was conducted using milk, with encapsulation done using enzyme-induced gellation. As with the other studies, survival rates of bacteria drastically increased after

incubation in gastric conditions when compared to the non-encapsulated bacteria (Heidebach *et al*., 2009). The proposed method of protection is that proteins have a buffering capacity, allowing little change in pH within the protein structure, preventing the lower gastric pH affecting encapsulated probiotic bacteria within the protein system (Heidebach *et al*., 2009).

2.2.4 Enhanced coatings for stability and physiological protection

Systems used for the delivery of probiotics include the use of coated beads, capsules and tablets. A study conducted by Krasaekoopt *et al*. (2004) showed the effectiveness of alginate beads in the delivery of both *Bifidobacterium* and *Lactobacillus* bacteria. The formulated beads were composed of sodium alginate and chitosan which were both coated and uncoated. Results showed that upon exposure to simulated human gastric and intestinal fluid with or without bile salt, the chitosan coated beads showed the highest survival rate of *Lactobacillus* bacteria and that the *Bifidobacterium* bacteria did not survive exposure to the gastric media, even when coated. Iannitti and Palmieri (2010) also stated that *Lactobacillus* bacteria do possess gastric-protective properties compared to other species of bacteria, which would result in the survival of *Lactobacillus* bacteria and not the *Bifidobacterium* bacteria. Kaushal and Shao (2006) have also stated that *Lactobacillus spp.* have the ability to survive exposure to gastric acid. A similar study was conducted by Brachkova *et al*. (2010) which showed the possibility of delivering different probiotic bacteria species through alginate beads. This study showed that alginate beads can be used to deliver probiotics effectively to the human intestine whilst providing protection for the bacteria against the harsh gastric environment.

2.2.5 Advancements through encapsulation

Studies have been conducted on the protection of probiotic bacteria using capsules formulated by an encapsulating process. The use of calcium alginate is common in the encapsulation of probiotics as it is non-toxic to the bacterial cells. The study conducted by Chandramouli *et al*. (2004) on *L. acidophilus* involved the encapsulated bacteria being subjected to human gastric conditions. The encapsulation of the bacteria was done using alginate at various concentrations. The survival of these bacteria was found to be dependent on the concentration of the alginate coat with the higher concentration leading to a larger amount of viable bacteria surviving (Chandramouli *et al*., 2004). Albertini *et al*. (2010) conducted a study on various polymers microcapsules and beads. Similar results were observed with large numbers of probiotic bacteria surviving incubation under simulated gastric conditions.

2.2.6 Delivery of probiotics outside the gastrointestinal tract

Newer studies have also been focusing on areas of probiotic delivery other than to the gastrointestinal tract. A pilot study conducted by Santiago *et al*. (2009) showed the possibility of probiotic delivery to the human vagina to treat or prevent vaginal bacterial and fungal infections. Naturally colonizing bacteria such as *Lactobacillus* bacteria are found in the uro-genital tract of females and assist, as with intestinal flora, in the prevention of colonization of potentially pathogenic bacteria and yeasts (Reid, 2000). The chosen delivery system was fast disintegrating starch pellets that were found to be an acceptable means of delivering probiotic bacteria to the human vagina. Further studies into the use of probiotic bacteria for the treatment of bacterial vaginosis through the use of a vaginal capsule have also shown positive results for this treatment and due to its site-specific delivery, it avoids the issue of gastric conditions and bile exposure (Ya *et al*., 2010). With traditional probiotic formulations focusing mostly on the delivery of probiotic bacteria to the intestine and colon, very few studies have shown the positive benefits of probiotic delivery to the oral cavity for the prevention of oral bacterial and fungal conditions. A study conducted by Bosch *et al*. (2001), showed the potential use of probiotic bacteria for the prevention of oral conditions such as gingivitis and peridontitis with positive results showing that probiotic bacteria can exert health benefits outside the intestine and colon and prevent the occurrence of orally experienced conditions and due to its site of delivery, the issue of cell viability due to physiological conditions is not experienced. The possibility of rectally administering probiotic formulations which avoids the gastric environment exists, with the ability of delivering a large variety of bacterial species to the colon. The risk of invasion, however, of pathogenic bacteria to the small intestine is increased due to the large amount of bacteria in the colon and within fecal matter (Mombelli and Gismondo, 2000).

2.3 Effectiveness of Pharmaceutical versus Food-Based Commercial Formulations

Whilst many studies have determined the unreliable variation amongst food-based commercial products, many agree that pharmaceutically-based delivery systems are still the most reliable in the delivery of probiotics. It is that reason for the inclusion of many pharmaceutically carriers suspended in commercial food products. Examples of this are the encapsulation of powdered bacteria to be delivered in yogurts (Kailasapathy, 2006). This study showed a significant increase in viable bacteria delivered to the intestine compared to the unencapsulated bacteria. Studies have also been conducted to determine the difference in viable numbers of bacteria when delivered as a capsule, in cheese or in milk. Saxelin *et al*. (2010) showed the comparison of cheese, capsules and yogurts for the delivery of probiotics by determining the amount of bacteria found in the feces of the participants after

ingestion of the respective delivery systems. It was also determined that the longer the bacteria took to appear in the fecal samples, the higher the degree of adhesion of the bacteria to the intestinal walls. Results from the study showed that the highest fecal bacterial count came from the yogurt product with a close comparison found between the yogurt and the capsules. The cheese product was found to have the lowest number of fecal bacteria and was determined to be the poorest delivery system of the three tested. The use of commercial food products in pharmaceutical products can be found to be beneficial for the delivery of probiotics. Capsules of probiotics were formulated using calcium alginate and Hi-Maize starch, which improved the encapsulation of viable bacteria compared to when the bacteria were encapsulated without the starch (Sultana *et al*., 2000). The encapsulated bacteria in this study, however, did not demonstrate a significant increase in survival when subjected to simulated gastric and intestinal conditions.

A preliminary study was conducted to monitor the effects of encapsulation on the survival of *L. acidophilus* and *Bifidobacterium* in yoghurt over a period of eight weeks (Sultana *et al*., 2000). The study showed that *L. acidophilus* and *Bifidobacterium spp*. exhibited a smaller decline in viable counts of about 0.5 log over the test period, while free cells had a decline of about 1 log in cultures, stating that encapsulation within the yogurt might prove beneficial to ensure survival of the bacteria in the product until the end of its shelf life (Sultana *et al*., 2000). From the studies that have been discussed, it can be noted that in the delivery of probiotic bacteria, due to the effectiveness and reproducibility of pharmaceutical formulations and the nutritional and microbiological value of commercial food-based products, the ideal probiotic formulation exists in the combination of these two broad groups of probiotic formulations.

2.4 Current Factors Affecting Probiotic Delivery

With the issues already highlighted in this chapter and the necessary changes which have been taken by researchers to overcome these problems, certain issues still surround the effectiveness and safety of probiotic formulations. Whilst recently, some researchers have questioned the effectiveness of probiotics in general to exert health benefits, the general consensus is that probiotics do supplement intestinal and uro-genital flora, which by themselves exert functional health benefits. The safety of probiotic formulations have also been questioned with studies finding probiotic bacteria in some formulations harboring antibiotic resistance, which can further precipitate life-threatening pathogenic conditions (Del Piano *et al*., 2006). A further issue raised has been that the numbers of probiotic bacteria in

formulations are so high that it may inadvertently prevent the detection of contaminants. A study performed by Joosten *et al*. (2005) which showed that current methodology for the detection of *Salmonella*, a potentially serious pathogenic bacteria, was ineffective in infant formulas due to the high numbers of probiotic bacteria present. This study highlighted that the tested infant formulas can potentially contain a variety of pathogenic bacteria with a false-negative result of non-contamination seen during microbial tests. There is also no guarantee that natural products including probiotics are free of contaminants, with cases of pathogenic bacteria, toxins and heavy metals being found in natural products (Penner *et al*., 2005). There is therefore a need for more stringent quality control in the preparation of probiotic formulations to ensure patient health and safety. *Enterococci* are a species of bacteria that have been linked to many pathological conditions in humans, with many having the ability to transfer antibiotic resistance onto other bacteria resulting in non-effective antibiotic treatment in many patients. However, some species of *Enterococci* have been found to treat gastroenteritis as well as not harboring virulent genes and not being resistant to vancomycin, the chosen treatment for methicillin-resistant *Staphylococcus* infections (Bhardwaj *et al*., 2010). While it has been shown that probiotic and health benefits may differ between species of the same genera of bacteria, many meat and dairy products containing *Enterococci* probiotics have been shown to harbor antibiotic resistance, placing the patient at a higher risk of serious infections whilst on probiotic treatment. With this issue of safety, micro-organisms in food now have to undergo screening tests to be regarded as safe for consumption by humans (Jankovic *et al*., 2010). Further studies will also need to be conducted to distinguish between potentially pathogenic and beneficial species of bacteria to increase the number of functional probiotics on the market.

2.4.1 Probiotic formulation efficacy

Issues that have been further raised in probiotic therapy are the effectiveness of many probiotic formulations on the market. Many formulations tend to deliver multiple strains of bacteria with many of them not being effective as probiotics and do not comply with the requirements of probiotic bacteria (Coeuret *et al*., 2004). Multiple strains in a single formulation have been shown to be ineffective in the supplementation of probiotic bacteria and have been found to be a major issue in probiotic therapy (Makinen *et al*., 2012). Another issue is the misconception that all bacteria of the same class have probiotic effects. Many formulations display that probiotic bacteria are contained within the product, but deliver ineffective bacteria for the supplementation of intestinal flora, with others delivering pathogenic bacteria capable of inducing antibiotic resistance as well as other pathological conditions during administration (Reid, 2000). Natural intestinal flora also varies from person

to person, from race or ethnicity as well as within age groups. Children tend to have higher levels of *Bifidobacterium* bacteria in their intestinal flora compared to older patients with many *in vitro* studies not taking into account host-specific factors that may affect the efficacy of the delivered probiotic bacteria (O'Brien *et al*., 1999). For this reason, it is important to know the intestinal flora of the patient being administered to ensure maximum health benefits for the patient (Grzeskowiak *et al*., 2012). It is therefore recommended that more formulations be specific to the type of patient being treated. Most feasible would be by age group, which would have maximum health benefits for the patient.

2.4.2 Probiotic safety

Lack of industry standardization as well safety issues have plagued the use of probiotics with many having a negative view on probiotics despite their health benefits (Kopp-Hoolihan, 2001). New legislation and regulations in many countries are, however, now requiring research and validity of bacterial probiotic cultures prior to administration as probiotics. This will ultimately lead to increased safety and effectiveness for all patients being administered with probiotic supplementation (O'Brien *et al*., 1999; Makinen *et al*., 2012). FDA regulations do not currently control the premarket approval of food or supplementary products including that of probiotics. Few products do show the effectiveness and state the health benefits of their products, with even fewer accurately proving the effectiveness of the formulation in human trials (Douglas and Sanders, 2008). It is thus up to the patient to read labels and do extensive research that absolves them from any misinformation. This is extremely difficult considering the trust that the common public give to medical formulations that are presumed to provide health benefits. Further education of prescribing doctors will also prove to be beneficial as they can provide vital information to patients about the effectiveness, misinterpretations and risks of taking formulations that have not been proven or tested.

2.4.3 Probiotic viability

One other issue in probiotic therapy is the use of unviable, inactivated, bacteria in many probiotic formulations. With this increase in formulations delivering unviable bacteria, a few studies have been conducted to determine the effectiveness of these bacteria as probiotics. Whilst a certain degree of functionality has been found for non-viable bacteria to provide health benefits to patients, the common agreement is that they are no substitute and are unlikely to be as effective as viable bacteria for the supplementation of intestinal flora. More research is therefore required into these types of bacteria to determine their effectiveness as probiotic formulations (Makinen *et al*., 2012).

2.4.4 Autoimmune induction

More research is also needed into the possible effect of probiotics on the induction of autoimmune conditions due to the immune-modulating effects of probiotic bacteria (Zhou and Gill, 2005). A study into the inflammatory effects of probiotic supplementation in mice genetically predisposed to the development of autoimmune conditions showed no significant increase in intestinal inflammation of mice supplemented with probiotics in their diet versus mice that did not, furthermore leading to the belief that probiotic bacteria can be used for its immune-modulating health benefits without the issue of side effects in patients with or predisposed to autoimmune conditions (Zhou and Gill, 2005). Whilst this *in vivo* study has shown that probiotic bacteria have little or no effect on immune-induction, the severity of the condition warrants more research to prove this phenomenon does not occur in humans.

2.4.5 Research outputs

With the large number of probiotic publications currently in existence, it has been documented that one of the greatest issues facing probiotic delivery is the lack of original research. Stevenson and Blaauw (2011) have shown that using a common publication search engine that approximately 26% of all probiotic publications are review articles, concluding that probiotic research has a large number of review articles, often repeatedly citing the same information, and therefore suffers from a deficiency in extensive original data as opposed to other research fields. With the many unanswered questions regarding probiotic effectiveness and safety, as well as the constant finding of more safe and effective probiotic bacteria, probiotic research still remains a largely untapped field to be undertaken.

2.4.6 Alternative analyses for the determination of effectiveness for probiotic delivery

With the inability of simple dissolution analysis to accurately determine the effectiveness of probiotic bacteria to adhere to the intestinal wall and to simulate competition with the natural intestinal flora of the human intestinal tract, models have been developed for the accurate simulation *in vitro* on the ability of probiotic bacteria to merge into the intestinal environment (Molly *et al*., 1993). Such an example of a model is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) which looks at the competitive issue between administered probiotic and the current natural flora in the intestinal tract (Alander *et al*., 1999). The SHIME is basically a series of vessels that each contains the microbial content of a different part of the human gastrointestinal tract that has been set up to simulate the conditions, fluids, oxygen content and microbial content of the respective portion of the gastrointestinal tract (Molly *et al*., 1993; Alander *et al*., 1999; De Boever *et al*., 2000). A schematic of the SHIME is depicted in Figure 2.3. Adhesion of the delivered probiotic bacteria to the intestinal wall is

also a vital issue if the bacteria have to provide functional health benefits for the patient. Unadhered bacterial cells would travel through the intestinal tract and would subsequently be eliminated from the body before it could provide any health benefits to the patient. Many *in vitro* studies do not test the adhesion properties of the delivery system and many *in vivo* studies test fecal matter that contain probiotic bacteria that may have not adhered to the intestinal wall. However, ways have been developed to determine the adhesion ability of delivered probiotic bacteria. One such option is to use fluorescent markers to test the ability of bacteria to adhere to the intestinal wall. A study conducted by Mare *et al*. (2005) showed that adhesion by *Lactobacillus* can be determined using fluorescence *in situ* hybridization which picked up and quantified the ability of *Lactobacillus plantarum* and *L. salivarius* to adhere to the intestinal wall of pigs. Other methods for adhesion quantification of delivered probiotic bacteria include intestinal cell lines, DNA probes and growths specific media, however, each of these require specialized equipment that may not be available to all researchers (Mare *et al*., 2005).

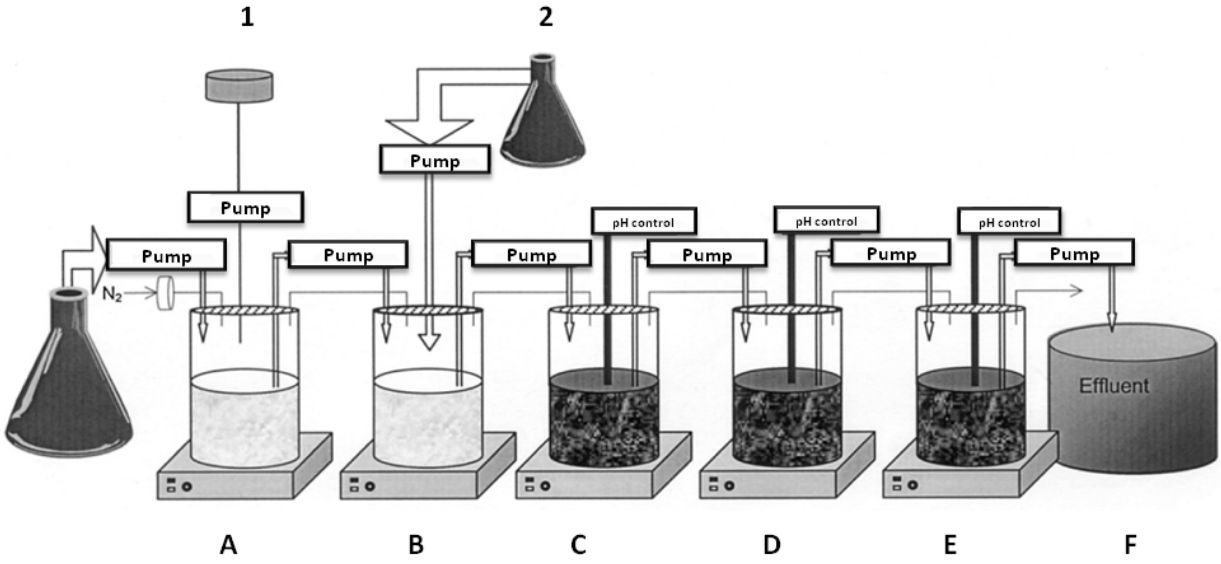


Figure 2.3: A schematic of an SHIME unit. Flasks 1-2 depict supplementary media for the system with vessels A-E depicting various areas of the intestinal tract and colon. These vessels are pH controlled using control units. Vessel F contains the effluent of the system. Image adapted with from De Boever *et al*. (2000).

2.5 Concluding Remarks

The efficacious delivery of probiotics has been a topic of interest for years with various approaches and systems being developed for the delivery of these bacteria to the intestinal tract. These systems are often pharmaceutically or naturally occurring food-based delivery

systems each having the capability of delivering adequate amounts of bacteria to ensure health benefits for the patient. However, with all the factors affecting probiotic therapy, it has been shown to be beneficial to have probiotic formulations that incorporate both pharmaceutical and commercial food-based excipients to have an effective dosage form for the delivery of probiotics to not only the intestinal system but as well as the uro-genital system. It is therefore hypothesized that formulation enhancement through the addition of energy-sources, prebiotics and vitamins, all of which is present in commercial food-based probiotic formulations combined with pharmaceutical products, that provide, amongst others, gastric-, cryo- and formulation protection, that the ideal probiotic formulation does not exist in the development of both fields of probiotic delivery but in a possible combination of the two. The formulated Dual-Biotic System of this study will therefore combine food-based ovalbumin with the required pharmaceutical modifications for the concurrent delivery of antibiotics and probiotics. Chapter 3 of this thesis provides the preformulation evaluation of a raw egg ovalbumin matrix for the delivery of *L. acidophilus* probiotic through the use of physicochemical, physicomechanical and stability analyses. The ovalbumin matrix forms the basic component of an advanced crosslinked delivery system that was utilized as the active therapeutic portion of the Dual-Biotic System. Further provided in the next chapter is the culturing and quantification protocol for *L. acidophilus* in addition to the mechanism of probiotic release from the ovalbumin matrix utilizing molecular docking studies.

CHAPTER 3

PREFORMULATION ANALYSIS OF AN ORAL OVALBUMIN-BASED DELIVERY SYSTEM FOR THE SITE-SPECIFIC DELIVERY OF PROBIOTIC *LACTOBACILLUS ACIDOPHILUS*

3.1 Introduction

Ovalbumin (of egg white origin) is known for its gelling, foaming and emulsifying properties in both the pharmaceutical and food industries (Kato *et al*., 1993; Mine 1996; Campbell *et al*., 2005; Talansier *et al*., 2009). Ovalbumin consists of large amounts of proteins, which can be defined as long chains of amino acids (Kato *et al*., 1993). The way in which these amino acids are structured determines their chemical and biological activity. Amino acids are naturally held together by weak non-covalent bonds, that when broken, are denatured (Cooper, 1999). Denaturing of ovalbumin, through chemical, thermal or mechanical denaturation, may therefore be advantageous as it allows for new structures with stronger bonds and different solubilities to be formed (Zemser *et al*, 1994; Cooper, 1999). Previously, ovalbumin in various dosage forms have been the focus of studies on vaccines with other research utilizing ovalbumin to induce a desired immunological response (Puri and Sinko, 2000; Pan *et al*., 2005; Boonyo *et al*., 2007; Misaka *et al*., 2009). The use of ovalbumin, however, for the delivery of other gastro-sensitive products, such as probiotic bacteria, has not been thoroughly investigated. The mechanism of gastric protection from ovalbumin has also not been determined with only a few studies focusing on determining the effect of pH buffers and chemical denaturation on the drug delivery properties of ovalbumin.

Proteins, such as ovalbumin, harbor an internal buffering capacity and upon the addition of an external pH, resists change to their own intrinsic pH value (Holma and Hegg, 1989). This ability of proteins can be manipulated to deliver pH-sensitive therapeutic agents such as probiotic bacteria, which are most viable when placed under their naturally occurring growth conditions. With regards to probiotic bacteria, they are also often anaerobic in nature and are most viable in conditions simulating the small intestine (pH 6.8) and colon (pH 7.2) (McConnell *et al*., 2008; Spinler *et al*., 2008). When expose to gastric conditions of pH 1.2, probiotic bacteria are often destroyed due to the harsh pH and enzymatic environment. *Lactobacillus acidophilus* has been shown in previous studies to harbor some resistance towards such harsh gastric conditions, however, this resistance is time dependent with *Lactobacillus* cell viability decreasing drastically after only a few hours (Maragkoudakis *et al*,

2006). The ability of *Lactobacillus* bacteria to integrate into intestinal and colon flora as well as the health benefits associated with *L. acidophilus* therefore warrants its use as the model probiotic in this study. The results obtained can furthermore be correlated to other probiotic bacteria.

In this component of the study, preformulation analyses were therefore undertaken on an ovalbumin matrix containing *L. acidophilus* probiotic, which was chemically denatured with PBS. For this purpose, the ovalbumin matrix was compressed into a minitablet that was thereafter subjected to *in vitro* probiotic release studies. Whilst, not the being the granule system utilized further in this study, the preformulation analyses conducted provided valuable data on the effect of chemical denaturation on the release of probiotic *L. acidophilus*, the protective properties of the ovalbumin matrix against compressive forces, as well as the mechanism of gastric protection and probiotic release. Additional preformulation studies undertaken in this chapter included the determination of the microbiological properties of *L. acidophilus*, the ideal form of ovalbumin to be utilized in the Dual-Biotic System and the effect of formulation processes on *L. acidophilus* delivery. The use of luminescence as a quantification tool for *L. acidophilus* was compared to common analytical quantification techniques such as Ultra-Violet Spectroscopy, with the limitations of each technique discussed and recommendations for their use in probiotic quantification provided as part of this chapter.

3.2 Materials and Methods

3.2.1 Materials

Lactobacillus acidophilus ATCC 314 was purchased from Davies Diagnostics (Randburg, Gauteng, South Africa). Raw egg ovalbumin was obtained from commercial chicken eggs (Nulaid®, Grade 1, Grain Fed, Johannesburg, Gauteng, South Africa). Purified ovalbumin derived from chicken eggs, pancreatin from porcine pancreas and pepsin from porcine gastric mucosa were purchased from Sigma-Aldrich (St Louis, MO, USA). MRS (de Man, Rogosa and Sharpe) broth, Thioglycolate broth, Thioglycolate agar and Tryptone Soya agar were purchased from Oxoid (Hampshire, UK). Skimmed milk was purchased from Clover® (Roodepoort, South Africa). BacTitre-Glo microbial cell viability assay was purchased from Promega (Madison, WI, USA). All other chemicals were of analytical grade and used as received.

3.2.2 Isolation, stock maintenance and quantification of *L. acidophilus* cells

L. acidophilus probiotic was cultured from lyophilized bacterial stocks. Briefly, the *L. acidophilus* cells were inoculated into 50mL of sterile MRS broth and incubated at 37℃ for 48 hours under anaerobic conditions in a candle jar. The bacterial cells were thereafter centrifuged (Lu Xiang Yi TDSA-WS, Shang Hai, China) at 3500rpm for 15 minutes, washed thrice with sterile 0.1% peptone solution and re-suspended in 10mL of sterile 10% skimmed milk solution. This solution was lyophilized at -80℃ for 24 hours (Labconco Freeze-Dry Systems, Labconco Corp., Kansas City, MO, USA) and thereafter stored under a desiccator at 4℃. The method development for culturing of *L. acidophilus* can be found later in Section 3.2.2.1 of this thesis. Purity studies were conducted on the MRS broth by placing the autoclaved media at room temperature overnight and sterility thereafter visually confirmed. Further purity studies were included in all microbiological analyses by placing the respective media at 37℃ for the duration of incubation.

Quantification of the viable bacteria present in the formulated lyophilized probiotic was necessary to ensure that the amount of viable probiotic bacteria required for therapeutic effects were maintained. Due to the large number of bacteria present in the formulated lyophilized *L. acidophilus* probiotic, a simple analysis through streaking of bacteria onto Thioglycolate agar plates would not suffice. Therefore, 10mg of lyophilized probiotic was added to 10mL sterile saline. Saline was used, as opposed to MRS broth, as the bacterial quantification medium, as it does not encourage bacterial growth and thus leads to more accurate results. Serial dilutions of 1:1000 were thereafter formulated by adding 100µL of stock solution to 9.9mL of sterile saline (Figure 3.1). The stock solution and dilutions (100µL) were thereafter inoculated onto Thioglycolate agar using the 'spread-plate technique'. This procedure was repeated thrice with the number of CFUs visually determined after incubation. This quantification analysis was conducted in triplicate to ensure accuracy of results and removal of any error that may have occurred during the dilution process. The amount of viable *L. acidophilus* cells present per milligram of formulated lyophilized probiotic was thereafter determined through back-calculation of the dilutions utilized. Figure 3.1 depicts the quantification process. Test tube No. 0 depicts the original stock solution formulated using a known concentration of lyophilized *L. acidophilus* probiotic (1mg/mL). Test tubes 1-4 depict the dilutions prepared with Test tube 1 containing the highest concentration of *L. acidophilus* bacteria and Test tube 4 containing the lowest. Sterility of the saline as well as all dilutions were determined by streaking onto Tryptone Soya agar (TSA) and incubating at 37℃ for 24 hours. This sterility procedure was repeated at all relevant

stages of this study to maintain accuracy of results and ensure no contamination had occurred.

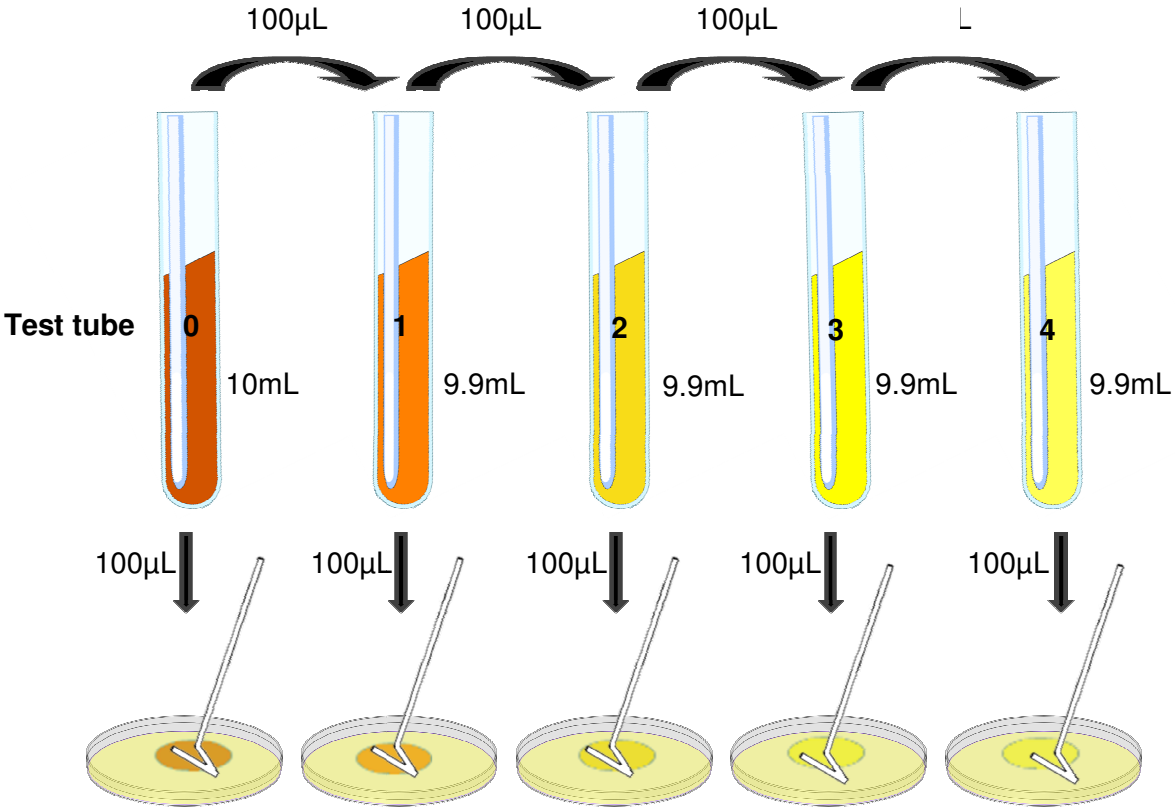


Figure 3.1: A schematic representing the quantification process for the determination of the number of viable probiotic bacterial cells present in the formulated lyophilized *L. acidophilus* probiotic.

3.2.2.1 *Method development for the culturing of L. acidophilus*

For the determination of the optimum culturing conditions required for the growth of *L. acidophilus* from the lyophilized bacterial stocks, MRS broth and Thioglycolate broth were evaluated. MRS is the commonly used media for the growth of *Lactobacilli*. However, Thioglycolate media was also analyzed due to its ability to facilitate growth of anaerobic bacteria (Engelkirk and Duben-Engelkirk, 2008). Both growth media containing culture were incubated in anaerobic gas jars and candle jars at 37℃ for 48 hours and analyzed for bacterial growth after incubation to ascertain an ideal growth environment to be utilized in the remainder of this study.

3.2.2.2 *Preparation of a calibration curve for the quantification of L. acidophilus*

To accurately determine the number of viable probiotic bacteria through luminescence, a calibration curve was constructed. Briefly, lyophilized *L. acidophilus* (10mg) was added to

100mL sterile distilled water to create a stock solution. A serial dilution was made by adding 0.1mL stock solution to 0.9mL distilled water to gain a final volume of 1mL. This procedure was repeated using 0.2, 0.3, 0.4 and 0.5mL stock solution diluted to 1mL creating a concentration range of 0.01-0.05mg/mL. An amount of 50µL of the mixtures were thereafter placed in individual wells of a 96-well plate with BacTiter-Glo Reagent equal to the volume of solution thereafter added to each well. The solution was thereafter mixed and incubated in an orbital shaker for 5 minutes. Luminescence of the bacterial ATP was recorded using a VICTOR X3 Multilabel Reader (PerkinElmer, Waltham, MA, USA) with a calibration curve being formed. This procedure was repeated in triplicate for accuracy.

3.2.2.3 *Construction of a bacterial growth curve for L. acidophilus*

A bacterial growth curve was constructed to determine the increase of bacterial ATP over time. For this, 50µL of MRS broth containing cultured *L. acidophilus* maintained at 4℃ was added to 10mL of sterile water. The solution of *L. acidophilus* was thereafter placed at 37℃ in an incubator with 50µL samples taken at hourly intervals. To determine the viability of the bacterial cells, BacTiter-Glo buffer (50µL) was added to the samples and the intracellular ATP determined through luminescence assay as described in Section 3.2.2.2.

3.2.3 Determination of the ability of raw egg ovalbumin and purified egg ovalbumin to act as a growth enhancer to delivered *L. acidophilus*

Due to the nutritional nature of ovalbumin, its ability for the growth enhancement of *L. acidophilus* was analyzed. For this analysis, two test tubes were incubated for 6 hours at 37℃ in an orbital shaker bath. Lyophilized *L. acidophilus* probiotic (10mg) was thereafter placed in each test tube, each containing 10mL sterile distilled water. Raw egg ovalbumin (0.5mL) and pancreatin were inoculated into one of the test tubes with 50µL samples removed after the incubation period. The other test tube was a control sample without ovalbumin. The procedure was repeated utilizing purified ovalbumin (10mg) with both analyses conducted in triplicate. The raw egg ovalbumin utilized was isolated from chicken eggs through separation of the egg yolk and albumin components and isolating the ovalbumin through rapid agitation of the albumin, centrifuging at 3500rpm for 20 minutes and thereafter filtering in a Buchner Funnel (Chibnall *et al*., 1943; Datta *et al*., 2009).

3.2.4 Preparation of denatured raw egg ovalbumin containing *L. acidophilus*

Chemical denaturation of proteins such as ovalbumin has been noted to influence its release properties and internal pH (Scopes, 1994). To determine this effect on probiotic delivery,

lyophilized *L. acidophilus* bacteria (10mg) was added to 2mL of raw egg ovalbumin. Phosphate Buffered Saline (PBS) (0.01M; 2mL) at pH 7.0 was added to the mixture, mixed for 1 minute, placed in a sterile glass petri-dish and lyophilized for 48 hours as described in Section 3.2.2. This procedure was repeated at pH values of 1.2, 3.0, 5.0, 9.0 and 12.0.

3.2.4.1 *Preparation of sterile 0.01M Phosphate Buffered Saline at different pH values*

Potassium dihydrogen orthophosphate (1.36g) was added to 1L of water to produce a 0.01M solution. Hydrochloric acid (1M) or sodium hydroxide (1M) was added to gain the desired pH value using a pH meter (Eutech pH 510, Cyberscan, Singapore). The potassium dihydrogen orthophosphate was placed under Ultra-Violet light overnight to sterilize the powder with the hydrochloric acid and sodium hydroxide diluted down with sterile distilled water and inoculated onto TSA to ensure sterility of the liquids.

3.2.5 Analysis on the effect of lyophilization on the ability of raw egg ovalbumin to deliver *L. acidophilus*

Further analysis of the effects of formulation processes on the ovalbumin matrix was undertaken to determine the effect of lyophilization on raw egg ovalbumin. *L. acidophilus* bacteria (10mg) was suspended in 2mL of raw egg ovalbumin and lyophilized. As a comparison, 10mg of *Lactobacillus* probiotic was suspended in 2mL of raw egg ovalbumin without the process of lyophilization. The lyophilized and non-lyophilized dosage forms were placed into hard gelatin capsules prior to *in vitro* probiotic release analysis. Moisture content analysis was conducted on the lyophilized raw egg ovalbumin system prior to conduction of the *in vitro* probiotic release analysis to determine the effect of moisture content on probiotic viability.

Moisture content was determined using a Karl-Fisher Volumetric Titrator (Mettler Toledo V30 Volumetric KF Titrator, Mettler Toledo Instruments Inc., Greifensee, Switzerland) consisting of Hydranal® Composite and methanol analysis medium under constant stirring at 25°C. The analysis media was placed within an isolated system containing a platinum electrode connected to a galvanometer in a dead stop endpoint circuit. Briefly, the titrator was initially allowed to remove all excess moisture contained in the test vessel to ascertain a baseline drift reading. After addition of 3.2mg of sample, the test analysis medium was mixed for 60 seconds after which the analysis was conducted until all moisture from the test sample had been titrated.

3.2.6 Preformulation evaluation of the denatured ovalbumin matrix

Prior to the development of the Dual-Biotic System, it was necessary to ascertain the ability of the denatured ovalbumin to successfully protect and deliver probiotic *L. acidophilus*. It was also of importance to determine the effect on bacterial stability over time due to the addition of an external pH to the ovalbumin matrix. To achieve this objective, the ovalbumin denatured with PBS at various pH values was subjective to *in vitro* probiotic release analyses in addition to in-process validation tests.

Whilst, ovalbumin granules were utilized as the active therapeutic component of the Dual-Biotic System, the initial preformulation analyses were undertaken on an ovalbumin minitablet system that was formulated prior to the granulation process. This was required to ascertain the protective properties of ovalbumin with respect to compression as a result of a tabletting or granulation process in addition to the stability and release of probiotic *L. acidophilus*. Furthermore, due to the size of the minitablet compared to granules, a change in matrix size due to swelling and/or erosion could be easily identified. This was therefore a useful tool in the determination of the mechanism of *L. acidophilus* release. To ascertain the correlation between the ovalbumin minitablet and granules, *in vitro* probiotic release analyses was also included as part of the preformulation analyses prior to advancement into the Dual-Biotic System.

3.2.6.1 *Preparation of ovalbumin containing L. acidophilus minitablets*

Raw egg ovalbumin containing *L. acidophilus* minitablets were formulated using a Mini-Tablet Press (Rimek Minipress- II MT, Karnavati, Gujarat, India). The amount of lyophilized ovalbumin containing 10mg of *L. acidophilus* probiotic as stated in Section 3.2.4 (~300mg), was placed in a tablet press die of 10mm diameter and compressed to a width of 1.87mm (+/- 0.03mm). The formulated ovalbumin minitablet was thereafter subjected to in-process validation tests prior *in vitro* probiotic release analysis.

As a control to the *in vitro* probiotic release analysis of the raw egg ovalbumin minitablets, purified lyophilized egg ovalbumin (120mg) was rehydrated in 2mL sterile water. This control was utilized to determine the effect of denaturation on the purified ovalbumin matrix and provide a comparison to the raw egg ovalbumin system. The result of this control therefore validated the use of the more cost-effective raw egg ovalbumin over purified egg ovalbumin. After hydration of the purified egg ovalbumin, denatured purified ovalbumin minitablets at various pH values between 1.2 and 12.0 were formulated as outlined in Section 3.2.4.

3.2.6.2 *Preparation of an ovalbumin granule system for the site-specific delivery of L. acidophilus*

The ovalbumin granule system that was included in the Dual-Biotic System was formulated through compression of the lyophilized ovalbumin containing 10mg of *L. acidophilus* probiotic into the ovalbumin minitablet as outlined in 3.2.6.1. The formulated minitablet was processed into granules through a modified dry granulation method utilizing a blender. Granule size was maintained below 700µm using of a sieve with the formulated granules placed within a hard gelatin capsule prior to *in vitro* probiotic release analysis.

3.2.6.3 *Determination of the matrix hardness of the raw egg ovalbumin minitablets*

The matrix hardness of the minitablets were determined using a calibrated Texture Analyzer (TA.*XTplus*, Stable Micro systems, Surrey, UK) fixed with a ball probe indenter of diameter 4.981mm, set at a return distance of 20mm, return speed of 10mm/sec, indentation depth of 0.1mm and a 5kg load-cell. The indentation hardness was represented by conversion to the Brinell Hardness Number (BHN) (kgF/mm^2) calculated as per Equation 3.1.

$$BHN = \frac{2F}{\pi D\left(D - \sqrt{D^2 - d^2}\right)} \qquad \text{Equation 3.1}$$

Where:

F = force generated from indentation (kg),

D = diameter of ball probe indenter (4.981mm) and

d = indentation diameter (1.397mm).

The indentation diameter was determined through the Theorem of Pythagoras (Equation 3.2) modified for use in a sphere (Figure 3.2)

$$a^2 + b^2 = c^2 \qquad \text{Equation 3.2}$$

Where:

c represents the hypotenuse and

a and b represent the adjacent and opposite sides of the right angle triangle respectively.

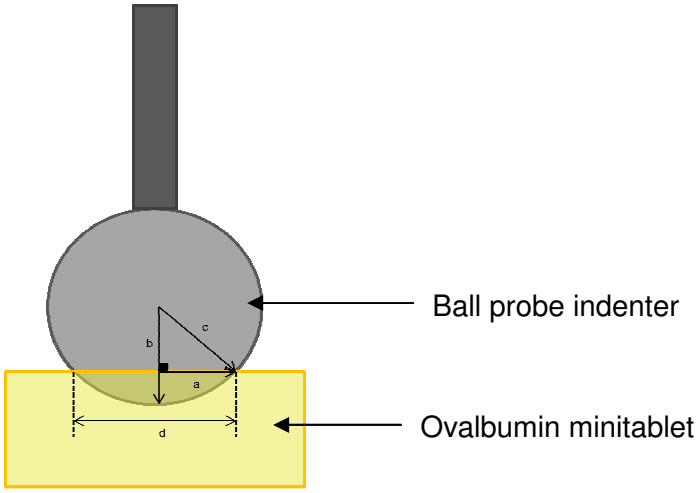


Figure 3.2: A diagrammatic representation of the use of the Theorem of Pythagoras for determination of indentation diameter [d] where (c) represents the radius of the sphere, (b) represents radius minus indentation depth and (a) represents half indentation diameter.

3.2.6.4 *Friability and weight uniformity of the raw egg ovalbumin minitablets*

Friability analysis on five pre-weighed ovalbumin minitablets was conducted using a Friabilator (Erweka D-63150, Heusenstamm, Germany) rotated at 25rpm for 4 minutes to constitute the 100 rotation required by the USP 33. After the 100 rotations, the minitablets were removed dusted and weighed to determine their weight loss after the friability test. The percentage friability was determined using Equation 3.3. The USP states that a friability of 1% is the upper limit of acceptability for tablet formulations. Weight uniformity analysis was also conducted on five ovalbumin minitablets by weighing each tablet using an analytical digital balance (Mettler, Model AE 240, Griefensee, Switzerland) with readings recorded to 2 decimal places.

$$WL_t = \frac{(W_0 - W_t)}{W_0} \times 100 \qquad \text{Equation 3.3}$$

Where:

WL_t is the total weight loss expressed as a percentage,

W_0 is the initial weight of the minitablets and

W_t is the final weight of the tablets.

3.2.6.5 *Determination of the viability of L. acidophilus in simulated gastric conditions*

With the determination of probiotic release being by luminescence, it was necessary to ensure that *L. acidophilus* was viable in gastric fluid prior to *in vitro* probiotic release analysis

in simulated human gastric conditions. This would ultimately determine that failure of the site-specific ovalbumin matrix, where bacteria are quantified in gastric media, could be determined through the use of luminescence. To determine the viability of the probiotic in simulated gastric conditions, 5mg of lyophilized *L. acidophilus* was incubated in simulated gastric fluid for 2 hours. Samples were collected per hour and analyzed using the luminescence protocol stated in Section 3.2.2.2.

3.2.6.6 *Preparation of simulated human gastric fluid*

Simulated human gastric fluid (SGF) was prepared as per USP guidelines. Briefly, 2g NaCl was added to 3.2g pepsin and 7mL hydrochloric acid diluted to 1L of autoclaved distilled water. The final solution was adjusted using hydrochloric acid to a pH of 1.2. Sterility of the SGF was determined through transfer onto TSA and incubation at 37°C for 24 hours.

3.2.6.7 *In vitro probiotic release analysis on the ovalbumin minitablets*

In vitro probiotic release analysis was performed on the lyophilized ovalbumin minitablets prepared in Section 3.2.6.1 using a USP II dissolution apparatus (Caleva 7ST, GB Caleva Ltd, Sturminster newton, Dorset, England) over a 12 hour period to ascertain the effect of pH denaturation of probiotic stability and release. Simulated Human Intestinal Fluid (SIF; 900mL; pH 6.8; 50rpm) formulated to USP specifications was used as the dissolution media. Pancreatin calculated to USP specifications of 1750 units of protease activity/1000mL was added to each vessel prior to *in vitro* probiotic release analysis. The minitablets were thereafter placed under ring-mesh assemblies to allow for complete minitablet surface area exposure to the dissolution media. All powders utilized in the preparation of the simulated gastrointestinal fluid were sterilized under UV light overnight with all liquids inoculated onto TSA and incubated for 48 hours at 37°C to ensure sterility. Samples (1mL) were withdrawn hourly from the release media and analyzed using the luminescence protocol stated in Section 3.2.2.2. Fresh buffer (1mL) was added to the dissolution media after sample aspiration to maintain sink conditions.

For the simulated transition of the ovalbumin minitablet through the gastrointestinal tract, the gastro-resistant ovalbumin minitablet was placed in dissolution vessels containing 200mL SGF for 2 hours. Samples (1mL) were withdrawn at hourly intervals. The minitablets were thereafter transferred into 900mL SIF, utilizing the 'teabag method' as outlined by Pourjavadi and Kurdtabar (2007), with modifications, for a further 12 hours. Briefly, tea bag paper with a 200µm pore size was used as the vessel for housing and transition of the minitablet across

simulated gastrointestinal media. The pore size of the teabag paper utilized was adequate to allow for transition of the bacteria and protein material into the dissolution media for sampling to occur. The time period of the release study was undertaken to determine the number of viable bacteria present in solution even after cessation of bacterial release from the ovalbumin minitablet. Due to the food-based nature of the delivery system, pepsin and pancreatin which readily digests proteins were added to the simulated gastric and intestinal media respectively (Fu *et al*., 2002).

3.2.7 Determination of the swelling and release properties of the gastro-resistant ovalbumin minitablet

Determination of the swelling and release properties of *L. acidophilus* from the ovalbumin minitablet was undertaken by exposing the minitablets to both simulate human gastric conditions, with and without pepsin, and simulated human intestinal conditions containing pancreatin utilizing the 'teabag method' as outlined in Section 3.2.6.7. The minitablet diameter and thickness were determined prior to and after the *in vitro* probiotic release analysis utilizing a digital caliper with the percentage increase in weight determined using Equation 3.4. The weighing procedure employed included removal of the hydrated ovalbumin minitablet matrix from the dissolution media and 'teabag', removal of the excess water through use of filter paper and weighing of the minitablet on a calibrated analytical scale.

$$\%\ increase\ = \frac{(W_t - W_o)}{W_0} \times 100 \qquad \text{Equation 3.4}$$

3.2.8 Magnetic Resonance Imaging of the gastro-resistant ovalbumin minitablet in simulated human gastric and intestinal fluid

A magnetic resonance system with digital MARAN-i configured with a DRX2 HF Spectrometer console (Oxford Instruments Magnetic Resonance, Oxon, UK) equipped with a compact 0.5 Tesla permanent magnet stabilized at 37°C and a dissolution flow through cell was employed for viewing of the swelling and release behavior of the gastro-resistant ovalbumin minitablet. After duly configuring, optimizing the shims and probe tuning, the cone-like lower part of the cell was filled with glass beads. The laminar flow of the SIF containing pancreatin was set at 16mL/min. The minitablet was thereafter placed in position within the cell, which in turn was positioned within a magnetic bore of the system. Magnetic resonance images were thereafter acquired every 10 minutes with MARAN-i version 1.0

software. MARAN-i software comprises of image acquisition software and image analysis software. The image acquisition parameters utilized are listed in Table 3.1.

Table 3.1: Image acquisition parameters applied during magnetic resonance imaging using MARAN-i.

Parameter	Value
Requested gain	4.17%
Signal strength	68.92
Average	2.00
Repetition time	1000.00 ms
Image acquired after	10 minutes
Total scans	144

3.2.9 Determination of the release mechanism of the ovalbumin minitablet

Due to the ovalbumin minitablet system being gastro-resistant in nature in addition to being able to swell and subsequently erode, it was necessary to determine the release mechanism of the delivery system. For this, ovalbumin minitablets were placed in simulated human gastric conditions for 2 hours to reach swelling equilibrium and were thereafter transferred (utilizing the teabag method outlined in 3.2.7.7) to simulated human intestinal conditions for 6 hours to erode. *In vitro* probiotic release samples were analyzed using UV Spectroscopy and the erosion rate constant calculated utilizing the Hopfenberg Erosion Model. This model was selected due to its ability to predict release from a surface eroding system (Dash *et al*., 2010). Deviations from the Hopfenberg Erosion Model were determined through further *in vitro* probiotic release analysis in simulated human gastric and intestinal conditions where the reduction in diameter and height of the minitablet was determined at hourly intervals utilizing a digital caliper.

3.2.9.1 *Construction of a calibration curve for the determination of L. acidophilus through ultra-violet spectroscopy*

For the construction of a calibration curve for *L. acidophilus*, 1mg of lyophilized *L. acidophilus* bacteria was added to 100mL of sterile water. Serial dilutions of 0.002, 0.004, 0.006, 0.008 and 0.01mg/mL were prepared and analyzed using UV spectroscopy (IMPLEN Nanophotmeter^{TM}, Implen GmbH, München Germany) at a wavelength of 208nm.

3.2.10 Molecular docking studies for the determination of enzymatic interactions between pancreatin enzymes and ovalbumin for probiotic bacteria release

To determine the mechanism of gastric-protection and subsequent release of probiotic bacteria from the formulated ovalbumin matrix in simulated intestinal conditions, computational modeling was utilized. Docking calculations were carried out using AutoDock (Bikadi and Hazai, 2009). This software includes code developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign (Humphrey *et al*., 1996). Gasteiger partial charges were added to the ligand atoms with non-polar hydrogen atoms merged, and rotatable bonds defined. Docking calculations were carried out on an ovalbumin conjugate-trypsin (PDB ID: 1QB1) model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools (Morris *et al*., 1998). Affinity (grid) maps of 20×20×20Å grid points and 0.375Å spacing were generated using Autogrid software (Morris *et al*., 1998). AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method (Solis and Wets, 1981). Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2Å, and quaternion and torsion steps of 5 were applied.

3.3 Results and Discussion

3.3.1 The ideal incubation conditions for the culturing of *L. acidophilus* cells

The number of viable bacterial cells obtained through incubation of anaerobic bacteria is largely dependent on the incubation protocol used as well as the media selected. It was therefore necessary prior to the lyophilization of *L. acidophilus* to generate an incubation protocol that would be reliable, time-saving and cost-effective that could be used for all microbiological analyses of *L. acidophilus* in this study. This therefore required a comparison of growth media and growth conditions for *L. acidophilus*. Results of the analysis revealed that MRS broth promoted the growth of *L. acidophilus* from bacterial stocks to a greater degree when compared to Thioglycolate broth. This can be attributed to the bacterial stocks being suspended in MRS during manufacture, leading to a shorter lag phase for the bacteria during growth. Further evaluation of the use of MRS agar and Thioglycolate agar, however, revealed that Thioglycolate agar was more beneficial for the visual quantification of *L.*

acidophilus. This was due to Thioglycolate agar causing an over-growth of *L. acidophilus* leading to larger CFUs being formed.

Anaerobic bacteria require an oxygen-free environment to grow. Two commonly used techniques were therefore evaluated, a candle jar which provides approximately 90% anaerobic conditions and an anaerobic gas jar which provides a virtually oxygen-free environment (Vinderola and Reinheimer, 1999; Alexandrakis *et al*., 2000). The ability of bacteria to grow in each of these jars varies within species of bacteria with strict (obligate) anaerobes, such as *Bifidobacterium*, needing total oxygen removal for growth to occur. *Lactobacillus* bacteria, however, being Gram-positive facultative anaerobes capable of utilizing oxygen if present for ATP production are able to grow in a candle jar even with its low oxygen content. Results of this analysis showed no significant variations in bacterial cell counts between the two anaerobic techniques. This lead to the candle jar being utilized for all microbiological culturing and quantification analyses due to its similar effectiveness as well as the cost-effective nature of utilizing candles over anaerobic gas packs.

3.3.2 Quantification of the cultured lyophilized *L. acidophilus* probiotic

Quantification from serial dilution determined that 1mg of lyophilized probiotic bacteria contained 1.73×10^7 viable *L. acidophilus* cells (n=3, S.D≤0.73) with Figure 3.3 depicting the results of the quantification process. Studies conducted have stated that the minimum number of probiotic bacteria to reach the intestinal system to ensure functional health benefits is approximately 10^8-10^9 probiotic bacterial cells (Doleyres and Lacroix, 2005; Kailasapathy, 2006; Oliveira *et al*., 2009). Therefore, to provide the ideal number of probiotic bacteria to exert functional benefits for the patient, 10mg of the formulated lyophilized *L. acidophilus* bacteria was to be added to all probiotic formulations.

The stated value of 10^8 probiotic bacterial cells is therefore referred to in this study as “target probiotic concentration” (TPC). This value however, does not indicate that when probiotic bacteria are released over a period a time, for example from a delivery system, that the delivered probiotic bacteria are sub-therapeutic. The stated minimum dose is the minimum number of viable bacteria required to be delivered to the gastrointestinal tract as a whole and therefore can be functional (in providing health benefits) even prior to this value being reached (Kailasapathy, 2006). The minimum dose will eventually have to be reached prior to exhaustion of the delivery system which will ensure functional health benefits to the patient.

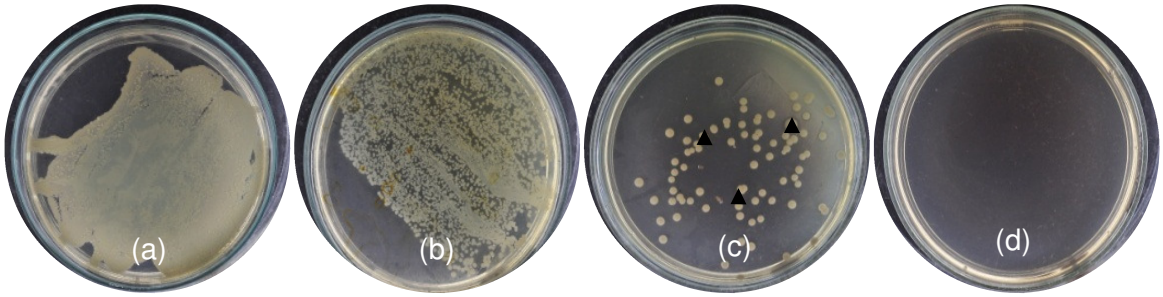


Figure 3.3: Digital images of the Thioglycolate agar plates inoculated with the serial dilutions as illustrated in Figure 3.1. Image (a) depicts the stock solution (Test tube 0) prepared, with images (b), (c) and (d) depicting the serial dilutions performed (Test tube 1, 2 and 3 respectively). The CFU counted after incubation are depicted using (▲).

3.3.2.1 *A luminescence bacterial growth curve for L. acidophilus*

A bacterial growth curve, which would determine viability of *L. acidophilus* over time, was necessary to determine the survivability of *L. acidophilus* in solution throughout the *in vitro* probiotic release analyses if no supplementary growth media was added. Cultured *L. acidophilus* bacteria stored at 4℃, which inhibits growth of bacteria, was added to sterile saline with samples taken at the respective time intervals to cover the initial lag phase, exponential phase, plateau phase and expiration phase that bacteria exhibit in bacterial growth curves. Lag phases are present in bacteria to allow for adaption to an environment. The exponential phase is where the bacteria have overcome the initial adaptation and becomes metabolically active in solution and is marked by a rapid increase in bacterial cell counts. The plateau phase is when all nutritional resources are exhausted by the bacteria and the subsequent death of the bacteria occurs afterwards in the expiration phase. A graphic representation of a typical growth curve in bacteria is shown in Figure 3.4.

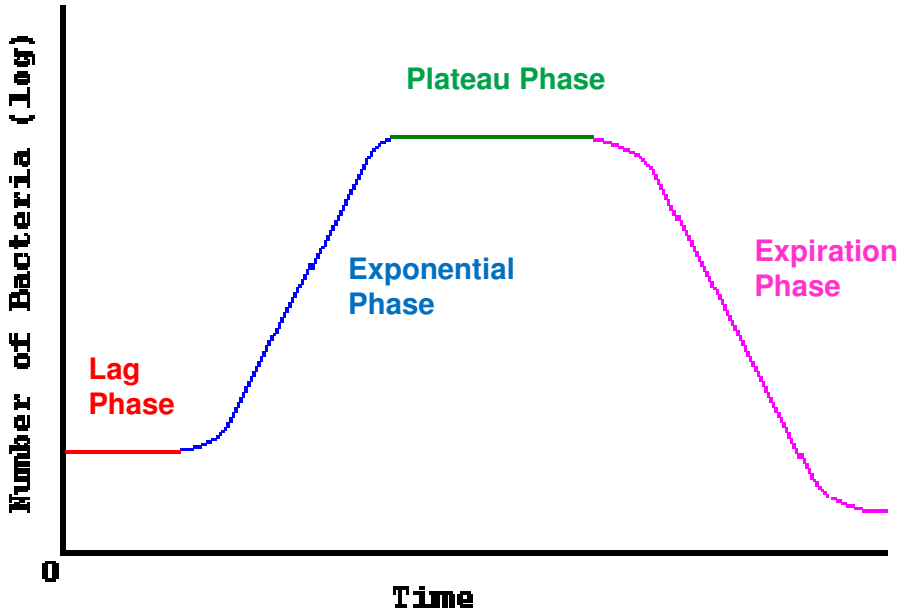


Figure 3.4: A typical bacterial growth curve. Adapted from Fowlkes (2005).

Results of this study showed an initial lag phase that occurred in a very short period of time between 0 to 1 hours, a short exponential phase from 1-3 hours and a plateau phase from 3-6 hours (Figure 3.5). The expiration phase occurred after 6 hours, where a rapid declination in bacterial ATP could be seen stating that the bacteria were no longer metabolically active and were subsequently dying in solution (Pelczar *et al*, 2010). The short exponential phase and plateau phase can be attributed to the lack of growth media in the sterile saline, which resulted in a short rise in bacteria cell counts and short stasis in solution respectively. From this analysis, it was determined that *L. acidophilus* would stay viable in solution during all in *vitro* probiotic release analyses that are conducted over a 6 hour period. This is of importance as the Dual-Biotic System would have a targeted maximum release of 6 hours after oral administration. The shortfall of this analysis, however, was the lack of growth resources in the solution during the course of this analysis, which was necessary to perform due to the length of release analyses and the metabolic nature of living viable actives such as probiotic bacteria. With regards to this study, this factor becomes important due to the carrier matrix of the delivery system which is protein in nature and could possibly act as growth media and growth enhancers to the loaded *L. acidophilus*. This will result in the plateau phase occurring at a much later stage than what has been seen in this analysis due to the utilization of growth media by the probiotic bacteria (Pelczar *et al*, 2010).

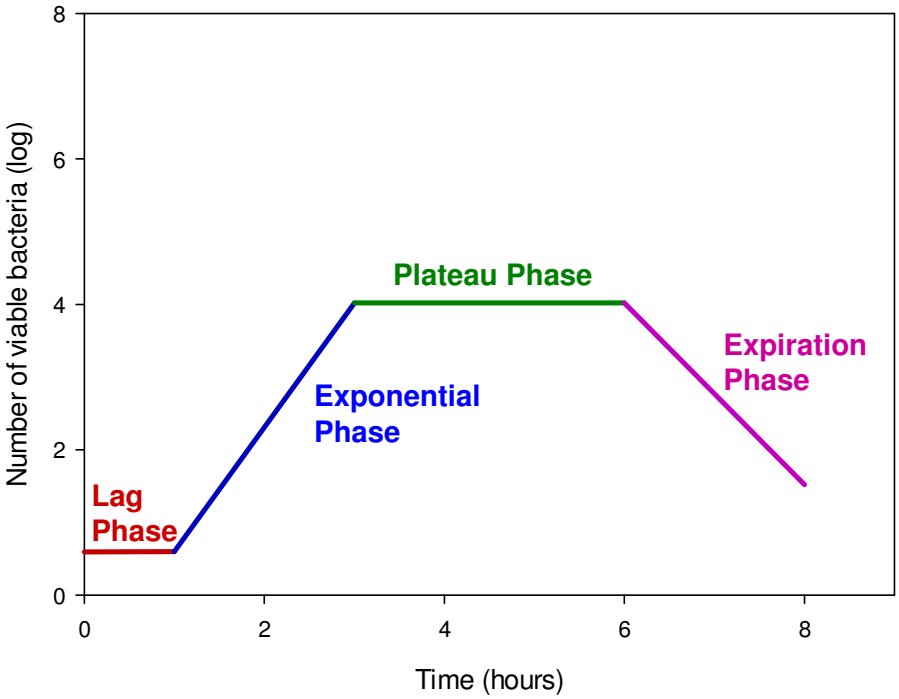


Figure 3.5: Generated *L.acidophilus* bacterial growth curve

3.3.3 The growth enhancing effects of raw egg ovalbumin and purified egg ovalbumin

With the addition of ovalbumin in the form of raw egg albumin, it was necessary to determine the ability of ovalbumin to provide supplementary media for the growth of *L. acidophilus* prior to the conduction of *in vitro* probiotic release analysis. The results of this study were vital as an increase in growth media for bacteria would increase their metabolic processes as well as increase their intracellular ATP resulting in inflated luminescence readings. It was also necessary to conduct this study as it also determined the ability of the ovalbumin carrier matrix to act as a growth medium once delivered to the intestinal tract. Results of this analysis have shown that ovalbumin does enhance the growth of *L. acidophilus*. Analysis of the use of purified ovalbumin showed a significantly larger increase in bacterial ATP concentration proving that ovalbumin does act as a growth enhancer. The difference between raw egg ovalbumin and purified ovalbumin for the enhancement of growth of bacteria, however, can be attributed to the lyophilized nature of purified ovalbumin that dissolves more rapidly than raw egg ovalbumin, allowing for quicker availability for the enhancement of growth compared to that of the raw egg ovalbumin. A control of lyophilized *L. acidophilus* bacteria in sterile water was added to create a baseline to which the ovalbumin sets were compared. Results of the analysis are shown in Table 3.2.

Table 3.2: Increases in bacterial viability when exposed to raw egg ovalbumin and purified ovalbumin.

Test Media	% Increase in viability
Raw egg ovalbumin	107
Purified ovalbumin	187
Control	0

Whilst the ovalbumin delivery system has been shown to provide growth media to the delivered probiotic, it can, however, not be classed as a conventional synbiotic delivery system. This is due to the criteria for the classification of a prebiotic. Prebiotics by definition cannot provide nutritional value to a patient which egg ovalbumin does (Schrezenmeir and de Vrese, 2007; Iannitti and Palmieri, 2010). Therefore even though the raw egg ovalbumin does provide growth media for the delivered probiotics, the system entirely cannot be classifies as a synbiotic delivery system.

3.3.4 A calibration curve for quantification of the lyophilized *L. acidophilus* probiotic

Through the construction of the calibration curve a luminescence constant of 275604.7614 at a R^2 value of 0.99 was obtained (Figure 3.6). This is a modification from Beer-Lamberts law which is used in the quantification of drug molecules via UV spectroscopy (FCC, 1981). The revised equation is still however applicable to probiotic bacterial quantification through luminescence due to the luminescence readings being directly proportional to the number of viable bacteria in solution. Therefore, through the use of the calibration curve constructed, the luminescence value for therapeutic effects to be given to the patient was determined to be 3062.28cps, (10mg of the lyophilized *L. acidophilus* in 900mL SIF). This value can therefore be directly utilized as the luminescence value of TPC for this study.

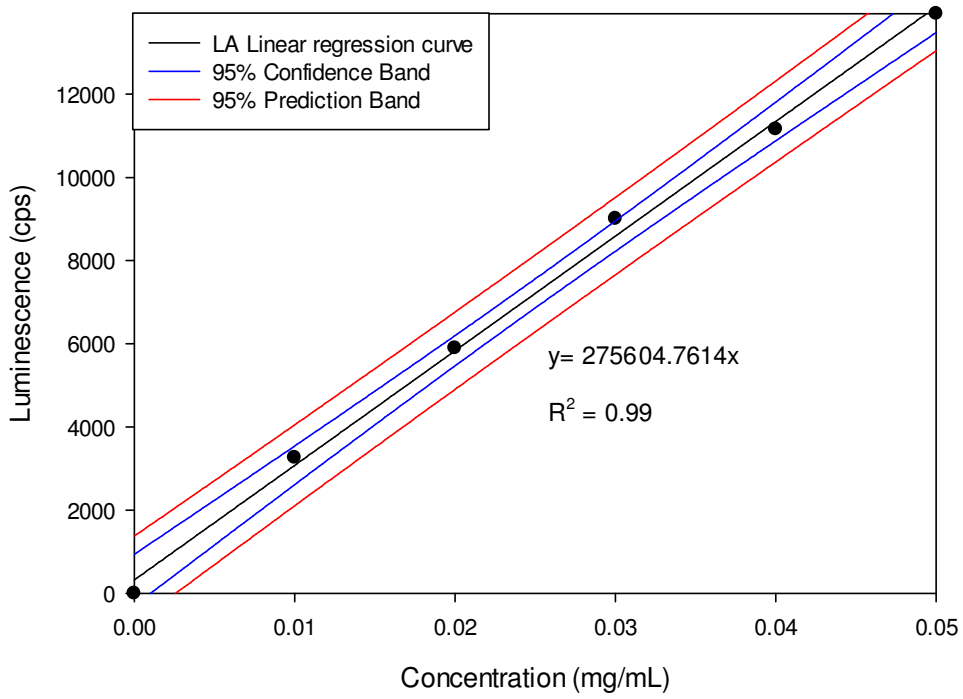


Figure 3.6: Calibration curve of *L. acidophilus* using the Bac-Titre Glo cell viability kit.

3.3.5 The effect of lyophilization on the ability of raw egg ovalbumin to deliver *L. acidophilus*

Similar probiotic release profiles between the lyophilized and non-lyophilized ovalbumin systems were observed during the release analyses (Figure 3.7). A minimal decrease in the viability of bacteria in the lyophilized ovalbumin system was seen and this can be attributed to the lyophilization process that can decrease the viability of the bacterial cell even with the addition of cryo-protectants. This is a well-known and reported phenomenon (Bolla *et al*., 2011). The non-lyophilized system showed a minimal increase in bacteria viability over the lyophilized system, however, the hydrated nature of this system results in a decreased stability of the formulation over time. As described in Chapter 2, Section 2.2.2, this would ultimately result in a decreased bacterial viability upon storage when compared to the lyophilized system and is therefore unsuitable for advancement into a probiotic delivery system.

The lyophilization process would also explain the initial lag in bacterial viability seen in the lyophilized system when compared to the non-lyophilized liquid form. The subsequent rise in the viability of the lyophilized system after 2 hours, to a similar viability to that of the non-lyophilized system after 12 hours can therefore be explained with the entrapped bacteria only becoming fully metabolically active after this time point. This is due to the lyophilization

process placing the probiotic bacteria in a dormant state resulting in a decreased bacterial ATP during the lag phase of bacterial growth (Section 3.3.2.1). The determined viability of the non-lyophilized system after 12 hours, which was expected to be significantly higher than the lyophilized system due to the high initial release of the system, can also be explained due to the decreased stability of the hydrated system. This resulted in a decrease in the viability of the bacteria before conduction of this analysis. The subsequent slowing of the increase in bacterial ATP after 6 hours (a decrease in bacterial growth) is due to depletion of ovalbumin growth media present in the release media. The resultant bacteria transit to the plateau phase of bacterial growth (approx. 10 hours) and would thereafter after undergo cell death during the bacterial expiration phase. This is however a distinct prolonging of the onset of the plateau phase of bacterial growth when compared to the bacterial growth curve generated in Section 3.3.2.1 and therefore validates the usage of a growth enhancing matrix for probiotic delivery.

From this analysis, it can be seen that lyophilization had no effect on the release profiles of the raw egg ovalbumin system, however, lyophilization is crucial for preserving the stability of the system as well as to maintain the viability of the administered probiotic bacteria. This stability, however, is directly related to the moisture content of the ovalbumin system. Karl-Fisher titration conducted on the lyophilized ovalbumin containing *L. acidophilus* determined a moisture content of 4.392% which is within the ideal range stated of 2.8-5.6% (Zayed and Roos, 2004).

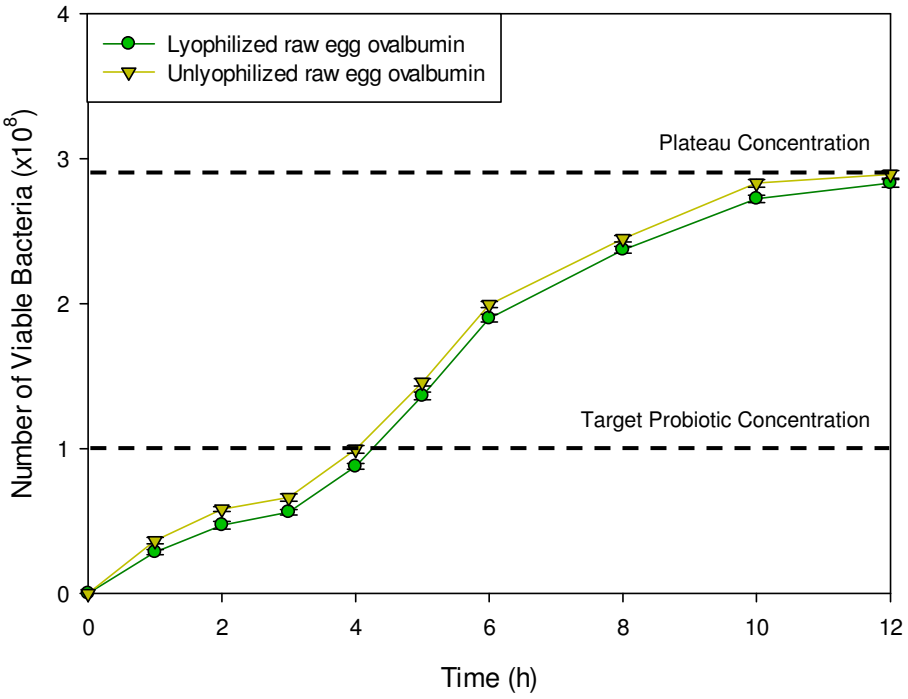


Figure 3.7: Probiotic release profiles of the lyophilized and non-lyophilized raw egg ovalbumin systems in simulated human intestinal conditions (n=3; SD ≤0.0268 in all cases).

3.3.6 Hardness, friability and weight uniformity testing of the raw egg ovalbumin minitablets

In-process validation tests revealed that the ovalbumin minitablets had a BHN value of 2.104kg/mm^2 (SD≤0.068), a friability of 0.2443% (SD≤0.079) and a weight uniformity of 237.42mg (SD≤5.48). The friability calculated is well within the upper limit of 1% as required by USP standards for tablets indicating that rigid, resilient minitablets were formulated. The weight uniformity of the minitablets had a small variation but still within acceptable limits.

3.3.7 Probiotic release profiles of the lyophilized raw egg and purified ovalbumin systems denatured at varying pH

From the values analyzed, pH 5.0 and 7.0 allowed for the highest viability after the tested time period. Less viability, whilst minimal, can be attributed to the extremes in pH, which is out of the optimal pH range commonly associated with *Lactobacillus* bacteria in the small intestine. Protein resistance to pH changes can explain the reason for pH 3.0 and 9.0 having significantly more viability as compared to pH 1.2 and 12.0. This is due to the resultant protein buffer capacity resisting large changes in pH. The probiotic release profiles of the pH values tested are shown in Figure 3.8a.

With the possibility of additives and contaminants present in raw egg ovalbumin, it was necessary to conduct the probiotic release analyses on purified ovalbumin to determine if there was a correlation. Consistencies between the two types of ovalbumin were noted with pH values of 1.2 3.0, 9.0 and 12.0 having significantly less viabilities when compared to pH 5.0 and 7.0.

The difference between the two types of ovalbumin is that purified ovalbumin had a significant burst of probiotic bacteria when compared to the raw egg ovalbumin. This can be attributed to the lyophilized nature of the purified ovalbumin prior to the formulation process. The lyophilized purified ovalbumin was reconstituted with sterile water to the concentration (60-65% total protein content and 10% chicken egg albumin) of ovalbumin commonly found in egg albumin. The subsequent removal of water through lyophilization resulted in the rapid hydration of the minitablet system on exposure to the *in vitro* dissolution media. This resulted in the high initial spike in probiotic concentration (Huntington and Stein, 2001). Furthermore, due to the lyophilized nature of the purified ovalbumin prior to formulation, the buffering ability of this type of ovalbumin was significantly less to that of the raw egg ovalbumin, resulting in pH changes having a greater effect on bacterial viability. This therefore resulted in the probiotic bacteria exposed to pH values outside their optimal ranges, which subsequently resulted in the inactivation of the bacteria to a greater extent. The probiotic release profiles of the purified ovalbumin denatured at various pH values are shown in Figure 3.8b.

From the probiotic release profiles generated, it was therefore noted that raw egg ovalbumin matrix provided greater bacterial protection from chemical denaturation than the purified egg ovalbumin matrix. It was also noted that the raw egg ovalbumin matrix provided a more controlled release profile when compared to the purified ovalbumin matrix. For this reason, purified ovalbumin was not utilized in the probiotic system as the rapid hydration and subsequent release of bacteria would expose the probiotic bacteria to gastric conditions when compared to the raw egg ovalbumin, where a stable matrix and much more controlled release was maintained. Therefore, from the pH values analyzed in the raw egg ovalbumin analysis, pH 7.0 was chosen to act as the denaturing buffer for the ovalbumin system during the remainder of the preformulation analyses, as this value provided a greater viability and more controlled release when compared to the other pH values analyzed.

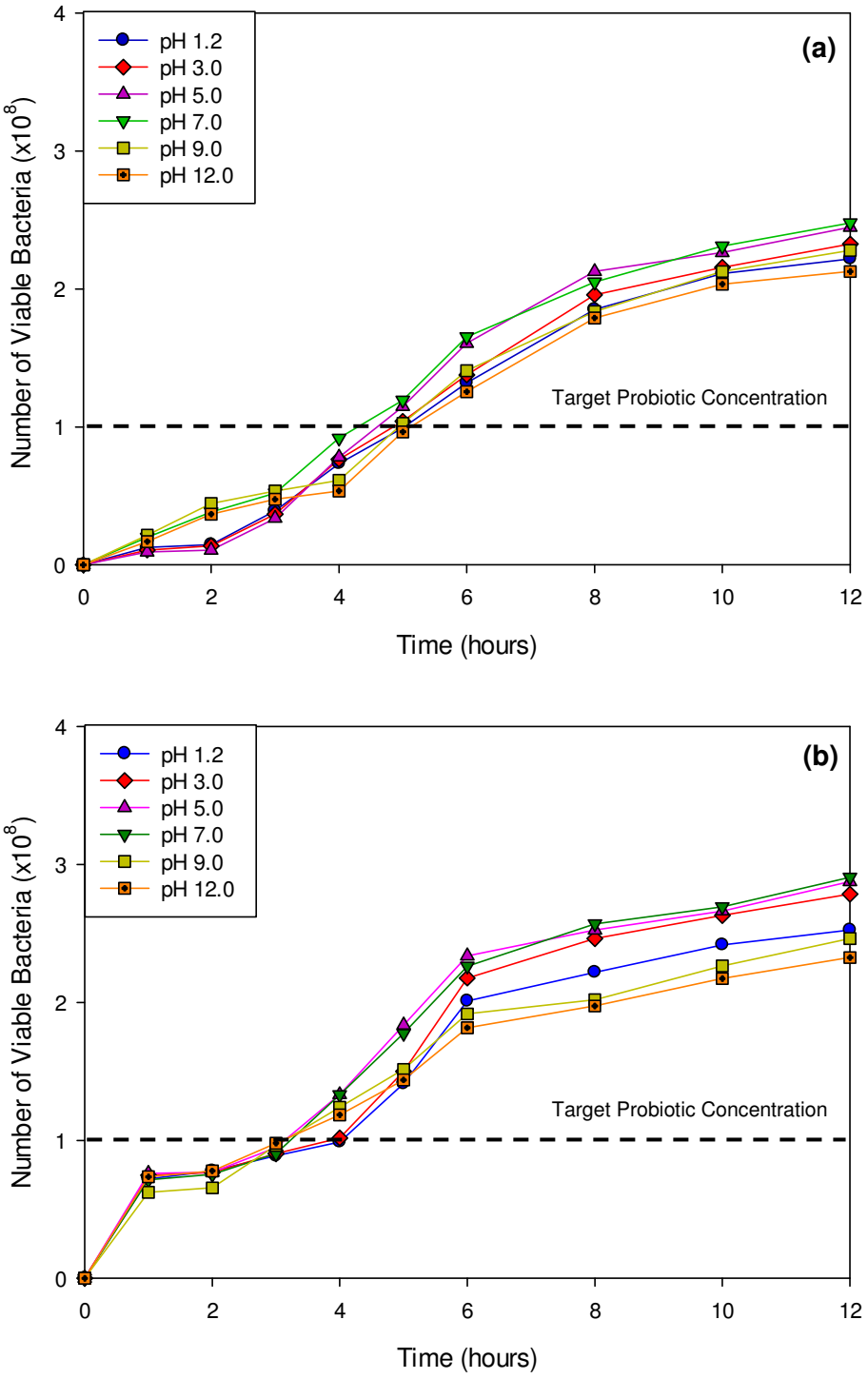


Figure 3.8: Probiotic release profiles of (a) raw egg ovalbumin and (b) purified ovalbumin systems denatured with PBS buffer at various pH values, (n=3; SD≤ 0.0431 in all cases). Maximum probiotic concentration = the maximum number of viable bacteria determined from all formulations analyzed.

3.3.8 The viability of *L. acidophilus* in simulated gastric conditions

After 2 hours in simulated gastric conditions, *L. acidophilus* showed considerable viability under the tested conditions (1394cps). The number of viable cells determined through calculation was, however, significantly less than the amount placed into solution (a luminescence of 3000cps as determined by the calibration curve formulated). This was consistent with other studies that showed that *Lactobacillus* bacteria has natural gastric resistance, but would eventually cease to be viable after a number of hours of exposure resulting in the subsequent death of all cells (Krasaekoopt *et al*., 2004; Iannitti and Palmieri, 2010). This result therefore showed that *L. acidophilus* would be viable in simulated gastric conditions (after 2 hours) and therefore its non-quantification in solution would accurately determine the gastro-resistant properties of the ovalbumin system.

3.3.9 Probiotic release of the ovalbumin minitablet in simulated human gastric and intestinal conditions

Probiotic release analysis of the ovalbumin minitablet determined a release of <10% of the loaded probiotic bacteria after 2 hours exposure to SGF with TPC reached within the 3 hours after transition to SIF (5 hours after onset of the release analysis). This was due to the molecular size of the probiotic being larger than the pore size of the matrix system in which no diffusion from the system was observed. The resultant delivery of probiotic was due to an initial burst-release as a result of the hydrodynamic pressure created just below the surface of minitablet. The effect of gastric media can also be seen with the ATP concentration remaining constant between 1 and 2 hours, where a decrease in bacterial cell counts prevented an increase in ATP with an increase in time. The effect of the ovalbumin buffering capacity can be attributed to the entrapped bacteria remaining viable despite being exposed to SGF.

Due to the initial swelling of the minitablet system in SGF and subsequent release in SIF, the loaded probiotic bacteria experience no lag phase when compared to the minitablet tested in the initial probiotic release analyses (Section 3.3.7). This lack in lag phase resulted in the production of ATP sooner. This phenomenon when combined with the growth enhancing effects of ovalbumin resulted in a greater luminescence count in SIF when compared to the results noted in Section 3.3.7. The release profile of the gastro-resistant minitablet exposed to SGF for 2 hours and SIF for a further 12 hours is shown in Figure 3.9.

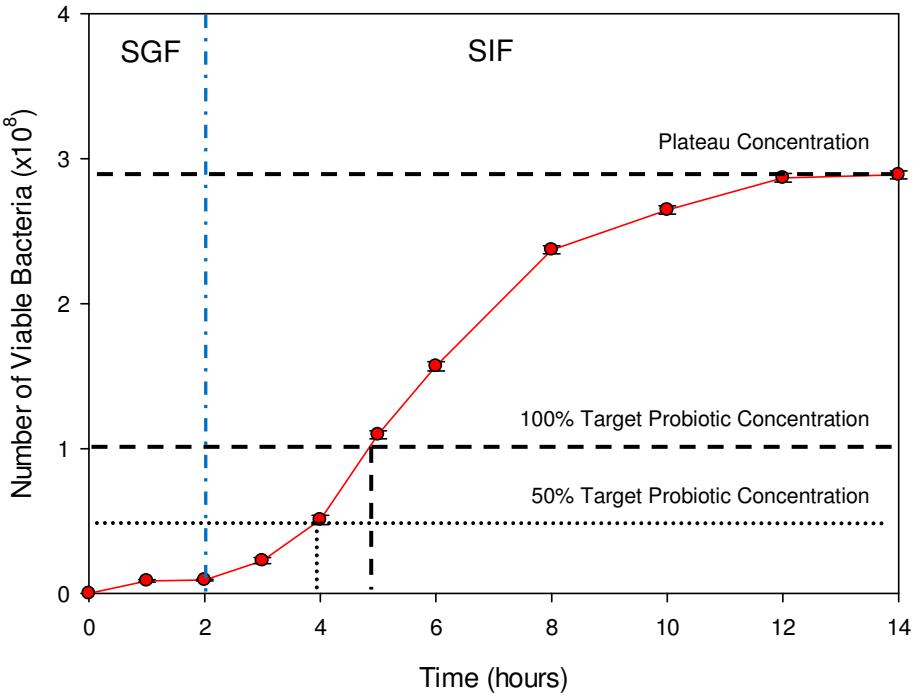


Figure 3.9: Probiotic release profile of the site-specific ovalbumin minitablet in simulated gastric fluid (SGF: 0-2 hours) and simulated intestinal fluid (SIF: 2-14 hours), (n=3; SD≤0.0331 in all cases).

3.3.10 Swelling and release behavior of the gastro-resistant ovalbumin matrix system

When placed in SGF without enzymes, the ovalbumin matrix swelled to an increased weight of 109%. With enzymes, swelling was once again noted but due to enzymatic degradation of the ovalbumin minitablet, the increase in weight was lower (75%). Pure ovalbumin harbors a natural resistance to enzymatic degradation by pepsin (Martos *et al*., 2010). The enzymatic degradation noted in this analysis can be attributed to other susceptible proteins present in the raw egg ovalbumin mixture utilized (Datta *et al*., 2009). When exposed to SIF, the ovalbumin minitablet swelled accordingly but was significantly smaller in size as compared to that of the minitablets exposed to SGF (Table 3.3). From this analysis, it was determined that the addition of enzymes as well as the pH of the dissolution media played an important role in the release of *L. acidophilus* from the ovalbumin matrix. Further analyses were therefore conducted to determine swelling of the ovalbumin minitablet in simulated gastrointestinal fluid as well as mathematical and molecular modeling to ascertain the mechanism of probiotic release.

Table 3.3: Tablet properties prior to and after exposure to simulated gastric and intestinal conditions.

Test media	Weight (0 hour) mg	Weight (2 hours) mg	% Weight increase (2 hours)	%Diameter increase (2 hours)	%Thickness increase (2 hours)
pH 1.2	299.9	627.9	109	154	281
pH 1.2 + pepsin	306.5	538.6	75	143	268
pH 6.8 + pancreatin	303.5	415.8	37	111	213

3.3.11 Magnetic Resonance Images of the gastro-resistant ovalbumin minitablet

Magnetic Resonance Imaging (MRI) of the ovalbumin minitablet visually determined minimal change of the minitablet core after exposure to SGF for 2 hours (Figure 3.10a-c). Minimal swelling had also occurred with an increase in size of the minitablet visualized after 2 hours. Upon exposure to SIF (Figure 3.10d-j), hydration of the minitablet can be seen over a 5 hour period with subsequent release of the encapsulated probiotic bacteria taking place. Visual assessment of the ovalbumin minitablet over a period of 24 hours revealed a slow decrease in the tablet surface leading to the possibility of erosion being hypothesized as the primary probiotic release mechanism from the ovalbumin matrix. This phenomenon warranted further investigation into the gastro-resistant properties of the ovalbumin matrix and subsequent mechanism of probiotic release in simulated intestinal conditions.

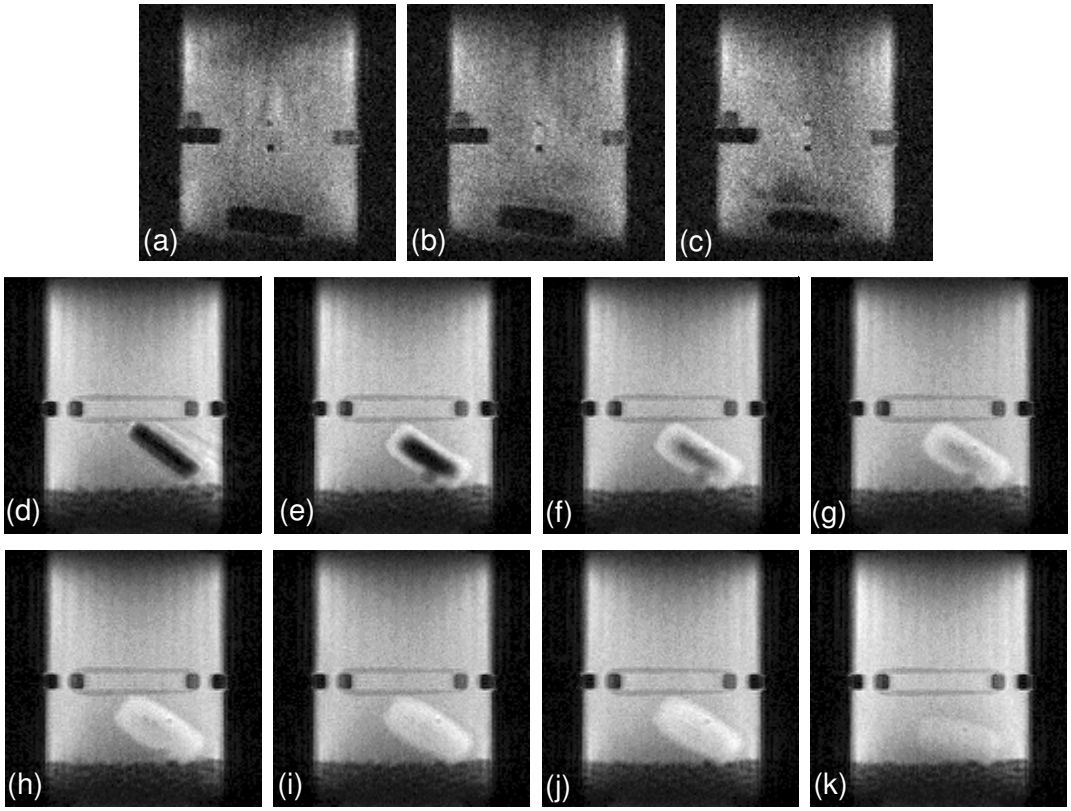


Figure 3.10: Magnetic Resonance Images of the ovalbumin minitablet in SGF after (a) 0 hours, (b) 1 hour, (c) 2 hours and SIF after (d) 0 hours, (e) 1 hour, (f) 2 hours, (g) 3 hours, (h) 4 hours, (i) 5 hours, (j) 6 hours and (k) 24 hours.

3.3.12 Calibration curve for *L. acidophilus* employing UV spectroscopy

An additional calibration curve for the determination of *L. acidophilus* concentration through UV absorbance was generated. While not being routinely quantified through UV spectroscopy, bacteria are known to be UV-active as a result of protein absorbance in the bacterial cell wall. The results of this calibration curve (Figure 3.11), showed an absorbitivity value of 19.7571 and a R^2 value of 0.99 when samples were analyzed at a UV wavelength of 208nm.

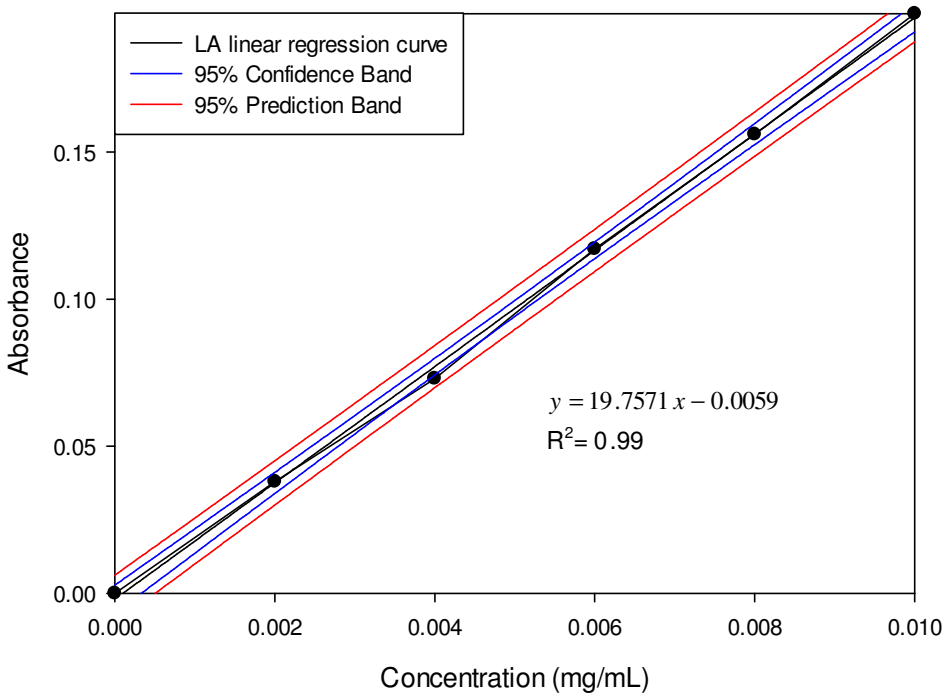


Figure 3.11: Calibration curve of *L. acidophilus* using UV spectroscopy at wavelength 208nm.

3.3.13 The mechanism of probiotic release from the ovalbumin minitablet

For the determination of the probiotic release mechanism, the Hopfenberg model (Equation 3.5) was employed. This mathematical model determines the release parameters of erosion-based systems (Costa and Lobo, 2001). The ovalbumin minitablet was proved to be such a system during evaluation of MRI results in Section 3.3.11.

$$\frac{M_t}{M_\infty} = 1 - \left[1 - \frac{k_o t}{C_o A_o}\right]^n \qquad \text{Equation 3.5}$$

Where:

$M_t / M\infty$ is the fraction of drug dissolved in solution,

k_0 is the erosion rate constant,

C_0 is the initial concentration of drug in the matrix,

A_0 is the initial radius of the cylinder or sphere or half the thickness of the slab and

n being the value of 1 for a slab, 2 for a cylinder or 3 for a sphere. A value of 2 was used for the ovalbumin minitablet system.

In vitro probiotic release analysis was conducted using UV spectroscopy where exhaustion of probiotic release from the ovalbumin minitablet system was determined. This was conducted to overcome the growth enhancing effects of ovalbumin which allows for luminescence increases after complete release from the delivery system. UV spectroscopy, determined the number of probiotic bacteria in solution, as well as cessation in probiotic release indicating complete release. The results of this analysis determined a large initial release of probiotic (Fractional Probiotic Release = ~0.52) from the minitablet system for the first 2 hours (Figure 3.12), gradually releasing up to 5 hours (Fractional Probiotic Release = ~0.83). The failure of the delivery system to reach a Fractional Probiotic Release (FPR) of 1 can be attributed to the cessation of enzymatic degradation due to the depletion of substrate as well as the increased stability of the system after 5 hours resulting in the prevention of further probiotic release.

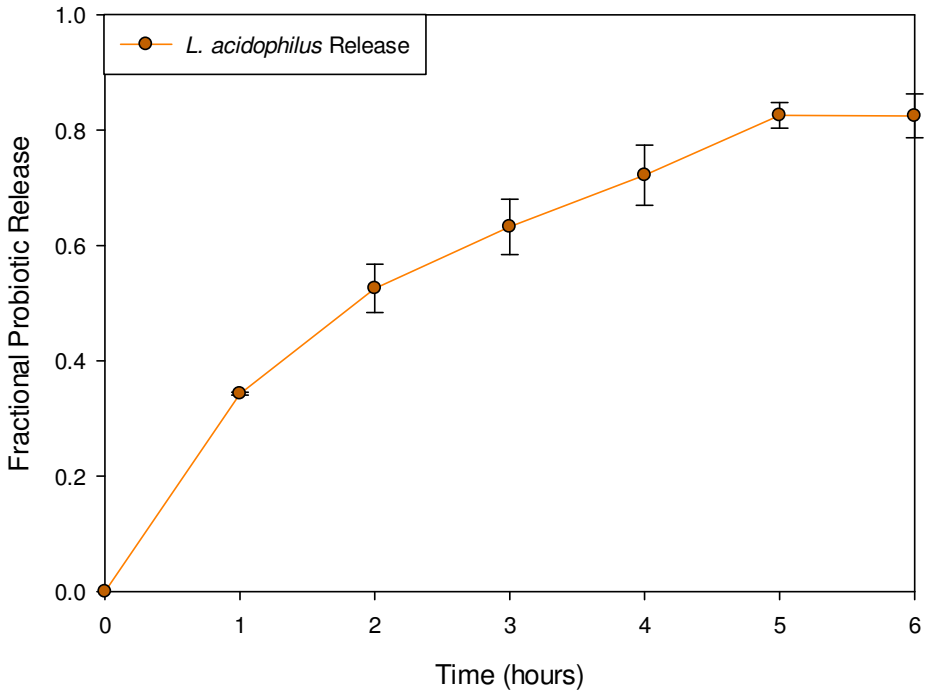


Figure 3.12: Probiotic release profile of the ovalbumin minitablet system using UV spectroscopy (n=3).

The mechanism of probiotic release analysis conducted on the ovalbumin minitablet system revealed an erosion rate constant of 2.66 and a R^2 value of 0.865. A linear regression curve was constructed detailing the accuracy of the erosion constants determined (Figure 3.13a). Further evaluation of the erosion rate constants revealed a hyperbola graph detailing a decrease in erosion rate constant as the ovalbumin system was present in the *in vitro* probiotic release media. There was a decrease in erosion rate constant which was constant

with an R^2 value of 0.989 revealing a deviation from the Hopfenberg model (Figure 3.13b). The erosion constants were thereafter calculated using the hyperbolic equation of y=1/x to determine the linear relationship of the decrease in erosion rate constant. Results of this analysis (Figure 3.14a) showed a linear relationship with a R^2 value of 0.997.

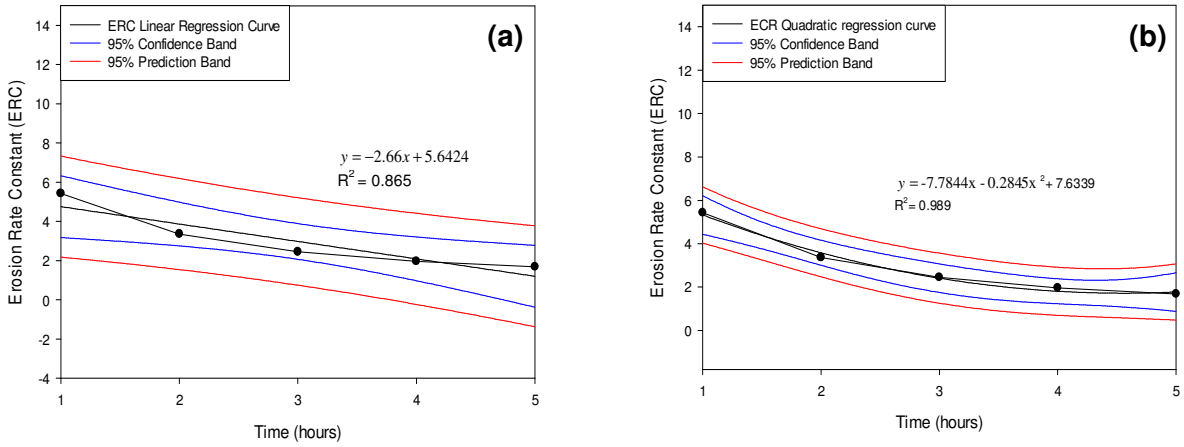


Figure 3.13: Graphical representations of (a) a Linear regression curve and (b) Quadratic regression curve of the erosion rate constant vs. time for the site-specific ovalbumin minitablet system.

To determine the reason for the deviation from the Hopfenberg model, the tablet radius and height were determined at each time point during the probiotic release analysis. The minitablet system was initially placed in SGF for the ovalbumin matrix to reach equilibrium prior to being placed in SIF. Results of this analysis showed a steady decrease in both the radius as well as the height of the minitablet system (Figure 3.14b). This can be attributed to bulk erosion that takes place in systems where enzymatic degradation occurs when the tablet system absorbs enough fluid to allow for digestive enzymes to enter the tablet matrix (Ratner and Hoffman, 2013). Therefore, from the analysis conducted, it was determined that the gastro-resistant ovalbumin minitablet system released probiotic bacteria through the process of bulk-erosion. This occurred at a constantly decreasing rate over time until complete probiotic release. Digital images of the ovalbumin minitablet prior to the analysis, after exposure to SGF and SIF are shown in Figure 3.15.

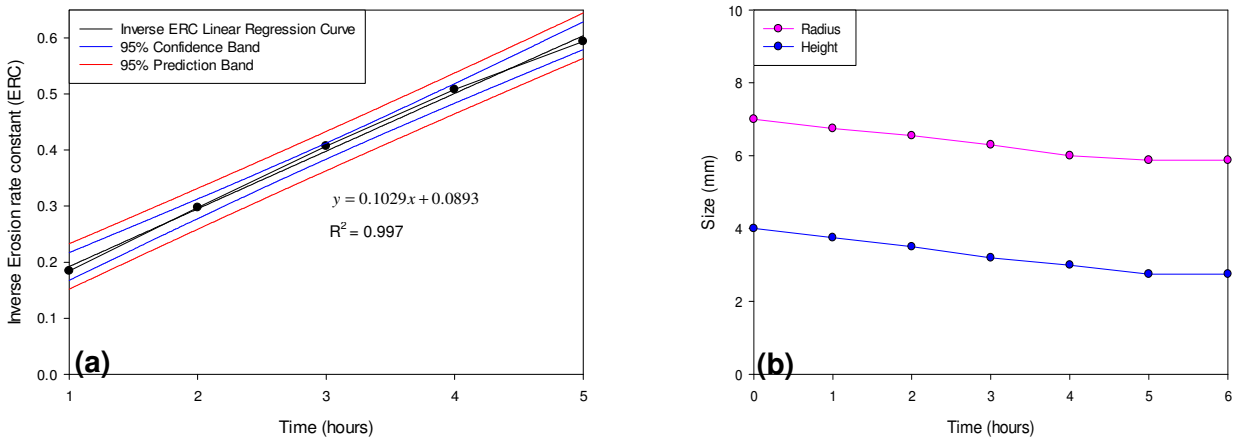


Figure 3.14: (a) a Linear regression curve of inverse erosion rate constant vs. time for the ovalbumin minitablet system and (b) the decrease in radius and height of the ovalbumin minitablet system in SIF.

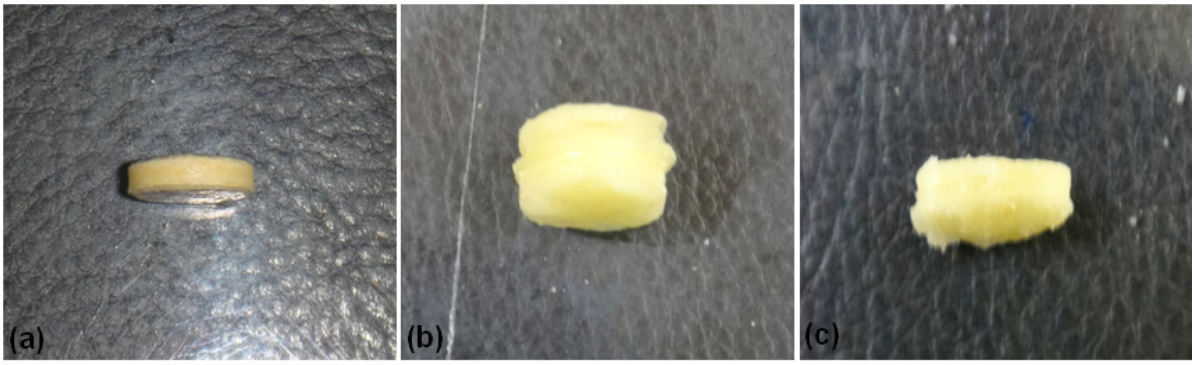


Figure 3.15: Digital Images of the ovalbumin minitablet (a) before conduction of the analysis, (b) after exposure to SGF and (c) after exposure to SIF.

3.3.14 Molecular docking analysis of the ovalbumin–pancreatic trypsin interaction complex (OPTiC)

To ascertain the effect of pancreatin protease–trypsin (PDB ID: 1QB1) on binding, cleavage, and degradation of the egg protein ovalbumin (NH_2-Leu-Gln-Ala-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-COOH), the molecular docking method was employed to simulate the binding mode between the component protein and enzyme structures. This was conducted to accurately determine the effect of pancreatin enzymes on the ovalbumin matrix to allow for the release of probiotic bacteria. Figures 3.16 and 3.17 display the best energy ranked docking results (established free energy of binding 6.95×10^{6}kcal/mol) and the interaction amino acid residues respectively.

The amino acid residues lining the OPTiC binding site comprise of Tyr20, Cys31, Ser34, Ile36, Val42, Ser43, Ala44, His46, Cys47, Tyr82, Ser84, Asn85, Thr86, Leu87, Asn88,

Asp90, Ile91, Ile109, Ser110, Leu111, Pro112, Ile124, Gln161, Met166, Phe167, Ser178, Ser183, Pro186, Val187, Val188, Lys192, Leu193, Ile196, Val197, Ser198, Trp199, Val211, Tyr212, Thr213, Lys214, and Val215 (Figure 3.17). Specifically, hydrogen bonds exist between the oxygen/nitrogen atoms at various positions of ovalbumin with Cys31, Ser34, His46, Tyr82, Ser84, Asn85, Thr86, Ser110, Ser178, Leu193, Ile196, Ser198, Trp199, Val211, and Thr213 of pancreatic trypsin. The folding/unfolding and conformational changes in the OPTiC were brought up by the polar amino acids Ser34, Ser43, His46, Tyr82, Asp90, Ser110, Ser178, Ser183, Trp199, Tyr212, and Thr213 making the complex interact well with the surrounding dissolution media. Importantly, the interactions representing enzyme catalysis, cation-π interactions, were represented by Tyr20, His46, Tyr82, Trp199, and Tyr212, confirming the position and conformation of the binding pocket responsible for the degradation of the ovalbumin matrix. Hydrophobic interactions (ovalbumin with Cys31, Ile36, Val42, Ala44, His46, Cys47, Tyr82, Leu87, Ile109, Leu111, Pro112, Met166, Phe167, Val188, Leu193, Ile196, Val197, Trp199, Val211, Tyr212, and Val215) and other forces (ovalbumin with Tyr20, Cys31, Ser34, Ile36, Val42, Ser43, Ala44, His46, Cys47, Tyr82, Ser84, Thr86, Leu87, Asn88, Asp90, Ile91, Ile109, Ser110, Leu111, Pro112, Ile124, Gln161, Met166, Phe167, Ser178, Ser183, Pro186, Val187, Val188, Lys192, Leu193, Ile196, Val197, Ser198, Trp199, Val211, Tyr212, Thr213, Lys214, and Val215) were also involved in the formation of OPTiC.

Considering the thermodynamic analysis of the molecular docking study, the total intermolecular energy was calculated to be 6.92×10^{6}kcal/mol, with electrostatic energy accounting for 3.04×10^{6}kcal/mol and combined van der Waals forces, hydrogen bonding, and desolvation energy contributing 3.88×10^{6}kcal/mol. The molecular docking study therefore revealed the effect of adding trypsin-containing pancreatin to the dissolution medium and the resultant enzymatic degradation of the constituent protein ovalbumin incorporated into the probiotic-loaded system. The resultant effect is the entrapment of the probiotic bacteria within the ovalbumin matrix in GIF, with release only occurring upon transition to SIF with trypsin-containing pancreatin. This result is in correlation with the in *vitro* probiotic release analyses conducted in Section 3.3.9 where the ovalbumin matrix was determined to be gastro-resistant in nature.

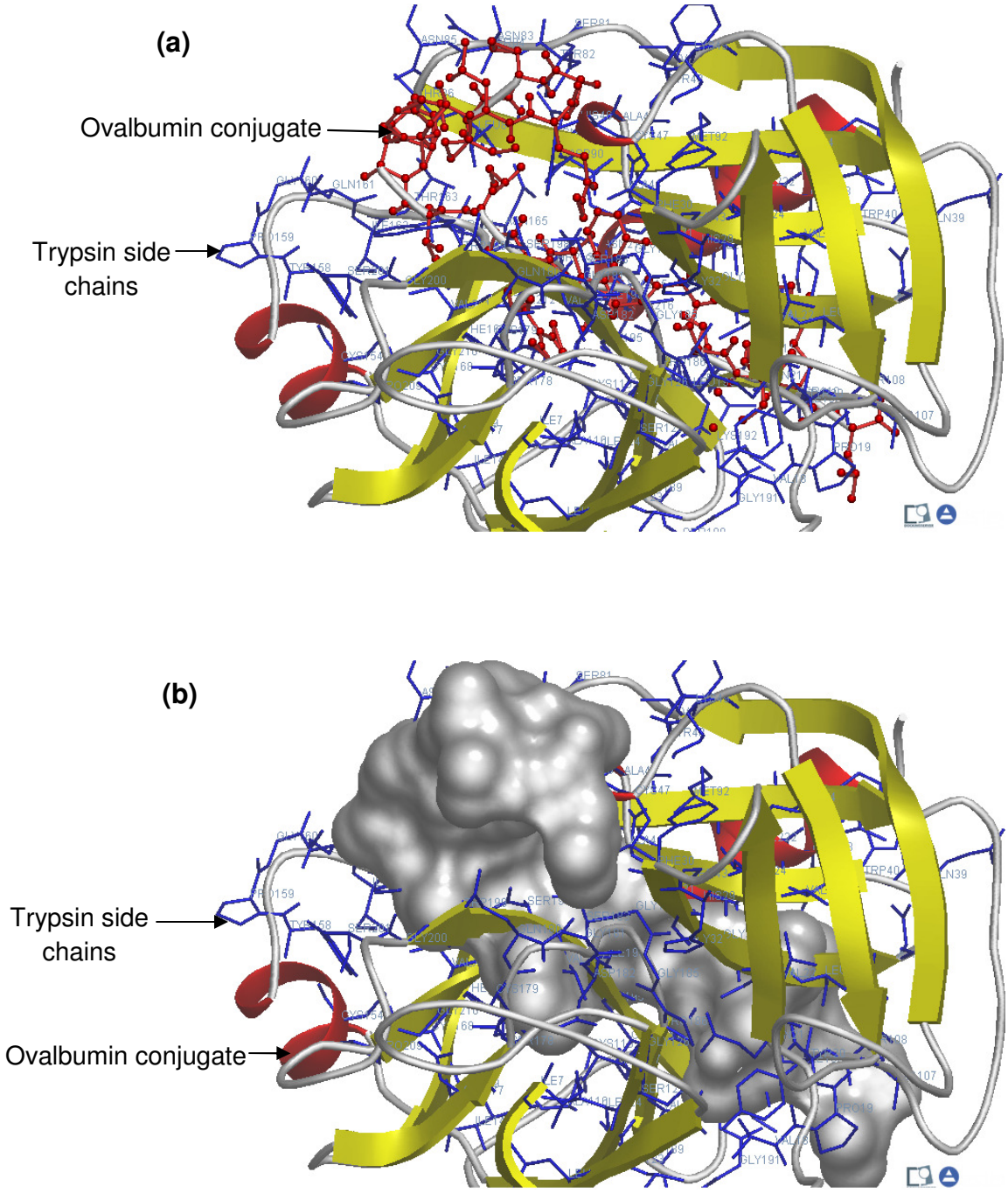


Figure 3.16: The binding mode between ovalbumin conjugate and trypsin, where the ovalbumin conjugate is depicted (a) as ball-and-stick model with red color coding; and (b) as surface mode. Trypsin is represented as tube-and-ribbon cartoon and the interacting side chains of trypsin are displayed in tube mode with blue color coding.

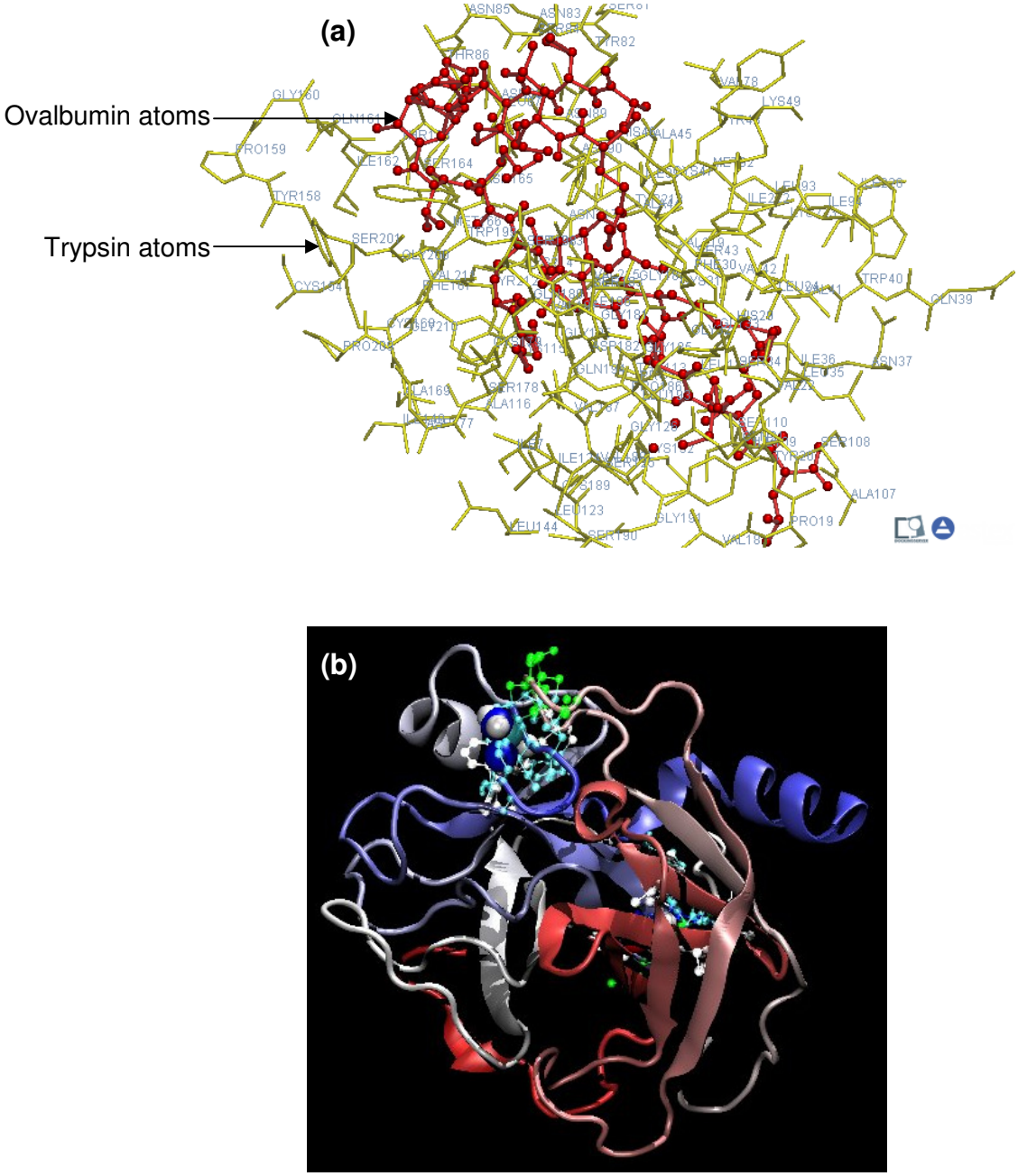


Figure 3.17: The molecular model showing (a) interacting amino-acids between ovalbumin and trypsin. The atoms of ovalbumin are marked with red and the atoms of amino acid residues of trypsin are labeled with yellow; and (b) the 3D-geometrical conformation of the ovalbumin–pancreatic trypsin interaction complex.

3.3.15 Comparative analysis on the use of luminescence and UV spectroscopy for the determination of *L. acidophilus* concentration released

Both luminescence and UV spectroscopy have been utilized in the preformulation study for the quantification of probiotic in solution. When the results of the *in vitro* probiotic release analysis of the ovalbumin minitablet were plotted onto the same profile (Figure 3.18), the characteristics of the two quantification techniques were determined. UV spectroscopy works well in determining the release profile of a chemical drug from a delivery system but fails to provide data on the viability of the probiotic bacteria. Luminescence provides data on viability and can also determine which cells remain viable in solution. This information can be accurately used in the determination of changes in cell concentrations in solution. Both techniques do, however, provide an increase in probiotic concentration over time indicating bacterial delivery (Figure 3.18). Between time period 0 hours and the point at which the plots intersect (approx 2.8 hours) probiotic bacteria is released, however ATP levels remain low. This is as a result of the lag phase of bacterial growth which takes approximately 3 hours until maximum ATP levels can be established. After 3 hours, the growth enhancing effects of the ovalbumin can be seen with ATP levels far exceeding the UV spectroscopy determination with the bacteria in the process of replication with UV spectroscopy not determining this phenomenon. Therefore from the analysis conducted, it can be concluded that both luminescence and UV spectroscopy can be used for the determination of bacterial concentrations in solution, however, when considering the use of either technique, the limitations of the technique should be considered to ensure accuracy of results.

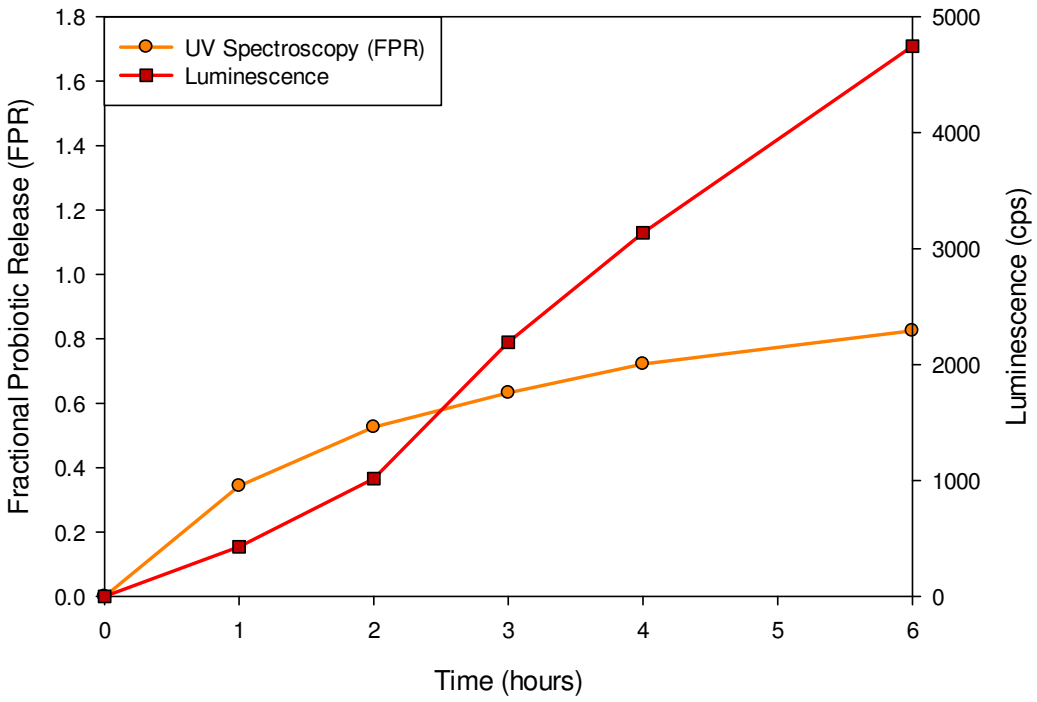


Figure 3.18: Comparison on the use of UV spectroscopy and Luminescence for the quantification of *L. acidophilus* bacteria released from the ovalbumin matrix.

3.3.16 *In vitro* probiotic release of the ovalbumin multi-particulate capsule system

With the use of ovalbumin for the delivery of *L. acidophilus* proven and evaluated in this preformulation chapter, it was necessary to determine if a correlation existed between the ovalbumin minitablet and granule formulations. Whilst the granule system, was derived from the simple breaking down of the ovalbumin minitablet to form particulates, the granule system was evaluated prior to advancement into the Dual-Biotic System.

Results of this analysis (Figure 3.19) revealed similar characteristics between the minitablet and granule systems for gastric-protection and probiotic release. It was, however, noted that the granule system did provide a greater initial probiotic release in both gastric and intestinal fluid, which can be attributed to the greater surface area of granules when compared to tablets. Apart from this characteristic, no significant difference could be determined between the minitablet and granule systems for the delivery of *L. acidophilus.* This result therefore validated the use of the granules in the Dual-Biotic system which would be more cost-effective to produce on a larger scale compared to the minitablet. This is as a result of the next step in the formulation process which is the formulation of the delayed release system, where melt coating with the use of glyceryl monostereate is employed, prior to inclusion into the Dual-Biotic System with amoxicillin trihydrate. Granules would also be significantly easier

to melt coat when compared to the minitablet which would require the formulation of a minitablet-in-tablet system (MTTS) to ensure adequate melt coating for a delayed release in addition to a rapidly disintegrating antibiotic layer. However, due to the positive results seen in the preformulation analyses, the MTTS was not completely discarded as a viable delivery system. The MTTS was advanced into a Bi-Layered Minitablet-in-Tablet System (BMTTS) for site-specific delivery to intestinal and colon conditions. This system can be utilized for intestinal and colonic flora deficiency and would require a formulation process with fewer steps than the Dual-Biotic System. The formulation and evaluation of the BMTTS is included in the end of this thesis in Appendix C.

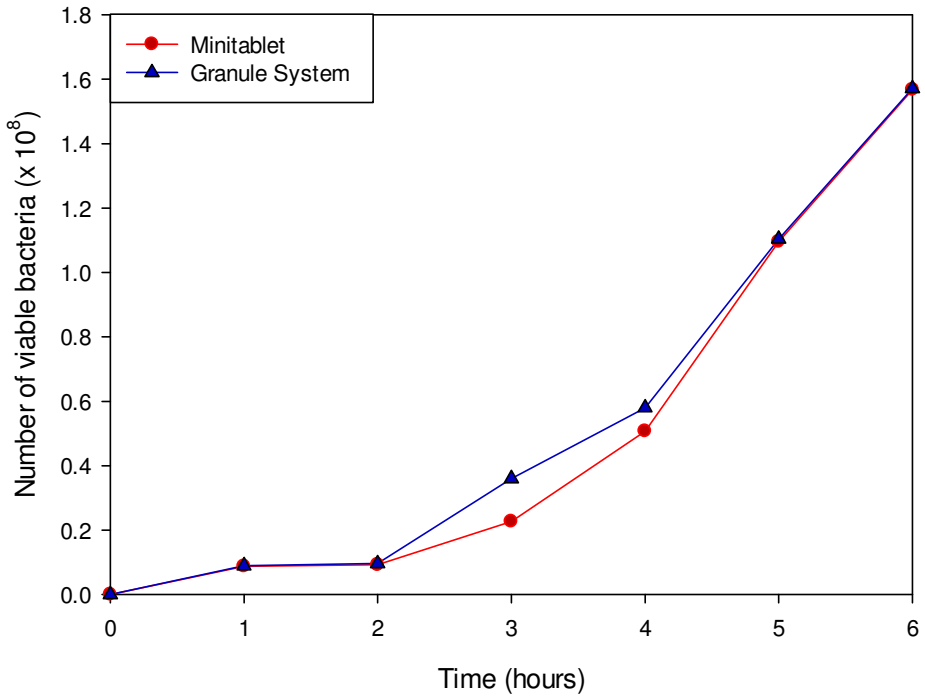


Figure 3.19: Comparative probiotic release profile of the ovalbumin minitablet and granule system for the delivery of *L. acidophilus* (n=3; SD≤ 0.064 in all cases).

3.4 Concluding Remarks

The denatured ovalbumin matrix has been successfully proven as part of this preformulation analysis to deliver (*in vitro*) *L. acidophilus* bacteria site-specifically to simulated human intestinal conditions. The use of molecular docking analysis predicted the effects of enzymatic degradation on probiotic release from the ovalbumin system, explaining the gastro-resistant properties of the ovalbumin matrix. It has also been noted in this preformulation chapter that lyophilization did not significantly affect the viability of the delivered bacteria. Luminescence has furthermore been shown to be an effective

quantification technique for probiotic bacteria. This can be a useful tool in conjunction with other analyses such as UV spectroscopy for future probiotic delivery systems which would ultimately benefit the patient and ensure the effectiveness of future probiotic delivery systems. The formulated ovalbumin matrix system was therefore advanced to formulate a denatured crosslinked ovalbumin granule system, which was statistically optimized in Chapter 4 for timeous probiotic delivery and thereafter advanced further to produce the delayed release and Dual-Biotic System in Chapter 5.

CHAPTER 4
DESIGN, FORMULATION AND OPTIMIZATION OF AN OVALBUMIN CAPSULE SYSTEM FOR THE DUAL DELIVERY OF ANTIBIOTICS AND PROBIOTICS (DUAL-BIOTIC SYSTEM)

4.1 Introduction

Probiotic therapy has been proven to be effective in the prevention of antibiotic-associated diarrhea, competing with potentially pathogenic bacteria for adhesion sites, ultimately preventing the colonization and functioning of these unwanted organisms. By their mechanism of action, antibiotics act on living bacterial cells when physical contact occurs. This therefore prevents the possibility of concurrent antibiotic and probiotic leading to the probiotic being administered two hours after the antibiotic. With preformulation studies conducted, raw egg ovalbumin has been shown to be effective in the delivery of probiotic bacteria to simulated intestinal conditions. An oral ovalbumin granule system was chosen to be the core active component that would be advanced further into a delayed release system that would be utilized as part of a Dual-Biotic System for the concurrent delivery of antibiotics and probiotics.

To achieve the development of a Dual-Biotic System, the ovalbumin granules would be required to possess certain criteria to allow for an effective formulation for the treatment of a patient. This required the use of a factorial design to statistically optimize the ovalbumin granules to set criteria. Prior to this, however, formulation issues seen in the preformulation phase of this study were to be overcome. Whilst the use of pH buffers having been shown to be advantageous in the delivery of probiotics, the rapid release of probiotic in the initial exposure to dissolution media was of concern. This initial burst result in a large number of probiotic bacteria delivered in the upper portion of the intestinal tract, which can be disadvantageous due to the requirement of the bacteria needing to be transported to the lower portions of the GIT by intestinal motility. This delay provides the opportunity for pathogenic bacteria to adhere to the intestinal wall binding sites that are now exposed due to the depletion of natural intestinal flora by the antibiotic. Whilst these pathogenic bacteria may be present directly after the depletion of intestinal flora or after a period time, the controlled release of probiotic bacteria throughout the intestinal tract prior to the administration of the next dose of antibiotic would be highly beneficial. Therefore to allow for a controlled release of probiotic bacteria, genipin was added to the formulation process. Genipin being a potent crosslinking agent that binds to methylamine or primary amine groups of proteins was

therefore utilized to control probiotic release from the raw egg ovalbumin system (Siritientong *et al*, 2013). Genipin, furthermore, when used in low concentrations would not result in the depletion of bacteria in the formulation process and was therefore preferred over other crosslinking agents of ovalbumin such as glutaraldehyde which has been used for cell fixing (Puri and Sinko, 2000). A chemical structure depicting the primary amines (NH_2) of ovalbumin is represented in Figure 4.1.

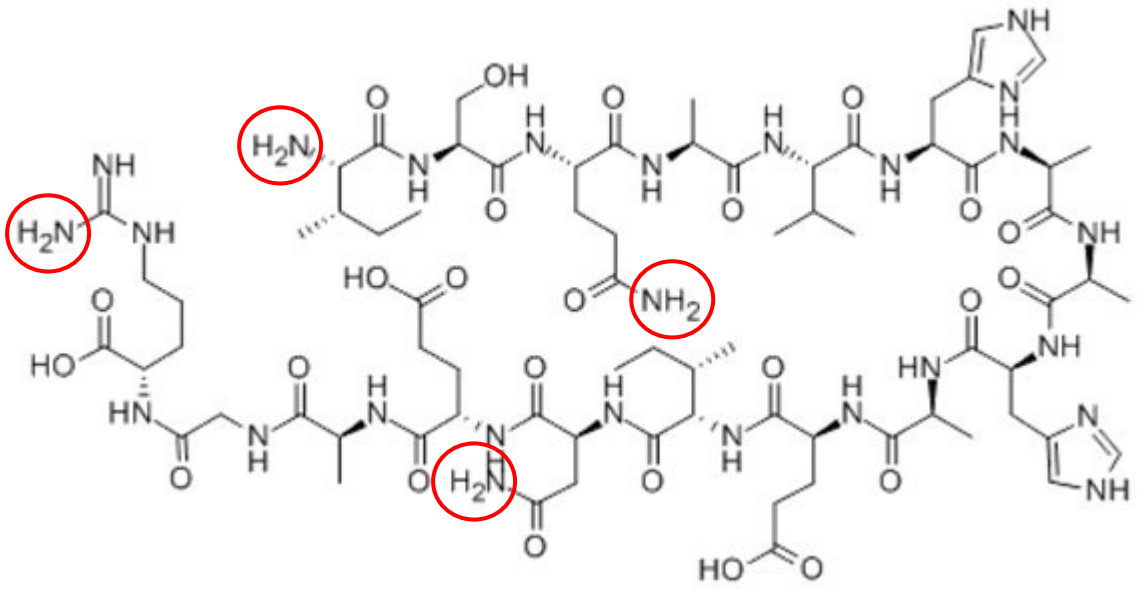


Figure 4.1: The chemical structure of ovalbumin.

With the addition of genipin, the ovalbumin granule system consisted of four components i.e. the probiotic bacteria, raw egg ovalbumin, pH buffer and the crosslinker genipin. These components would therefore form part of a design of experiments for the optimization of the granule system. For the design of experiments, a response surface-methodology approach utilizing a Box-Behnken experimental design was selected. This incomplete 3-level factorial experiment when utilized with three factors (variables) generates 15 formulations arranged along a low, medium and high scale in accordance with the variable parameters selected (Figure 4.2). This therefore results in a duplication of a single formulation occurring at the midpoint of the design (Red Dot) leading to a good estimate of the variance (standard deviation) within the experimental design (Box and Hunter, 1978).

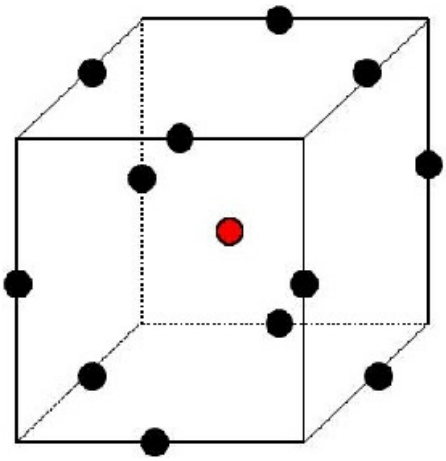


Figure 4.2: A 3-dimentional diagrammatic representation of the 3-level, 3 factor Box-Behnken experimental design highlighting the various formulations (back dots) and the design midpoint (red dot). Image reproduced from Prism TC (2008).

In order to conduct the statistical optimization of the granule system through the use of the Box-Behnken experimental design, the following actions were outlined:

- Identification of formulation components that can be utilized as variables in the construction of the factorial design.
- Identification of the variable parameters for the construction of the factorial design.
- Construction of the factorial design based on the variable parameters.
- Analysis of the formulations utilizing set responses.
- Regression analysis for coefficient evaluations.
- Response prediction and validation for statistical optimization.
- Statistical optimization of the granule system to a set protocol.

The design variables chosen were that of the formulation components that could be modified (*L. acidophilus* amount could not be altered due to the numerical requirement for therapeutic effects as highlighted in Chapter 3). These variables therefore included the amount of ovalbumin, the concentration of genipin and the pH of the denaturing solution. The design responses were chosen as per therapeutic and formulation requirements and will be highlighted in more detail. This chapter therefore provides the statistical optimization of the ovalbumin granules utilizing a Box-Behnken experimental design to set formulation and therapeutic considerations. Briefly, the initial component of this chapter highlights the determination of the design variable parameters for construction of the experimental design. The conduction of the design is thereafter undertaken with statistical analysis of the design responses performed in addition to the statistical optimization of the granule system.

4.2. Material and Methods

4.2.1 Materials

Raw egg ovalbumin obtained from commercial chicken eggs (Nulaid®, Grade 1, Grain Fed, Johannesburg, Gauteng, South Africa). Genipin, pancreatin from porcine pancreas and pepsin from porcine gastric mucosa were purchased from Sigma-Aldrich (St Louis, MO, USA). BacTitre-Glo microbial cell viability assay was purchased from Promega (Madison, WI, USA). All other chemicals were of analytical grade and used as received.

4.2.2 Preparation of crosslinked denatured ovalbumin granules for the delivery of *L. acidophilus*

The crosslinked denatured ovalbumin granules were prepared utilizing the granulation method as stated in Chapter 3, Section 3.2.6.2, with modifications. Briefly, *L. acidophilus* probiotic (10mg) prepared in Chapter 3, Section 3.2.2, was added to ovalbumin and mixed prior to the addition of pH buffer at the same volume. This solution was thereafter mixed for a further minute. Genipin solution was thereafter added to the ovalbumin solution which was mixed for a further 5 minutes. All solutions were kept at constant volumes to ascertain the effects of change in concentrations of ovalbumin, genipin and pH buffer during the experimental design. The resultant solution was thereafter frozen at -80ºC and lyophilized. The granules were formulated by the dry granulation method where tablet slugs were formulated and broken down to form granules. These granules were thereafter placed in hard gelatin capsules prior to analysis.

4.2.3 Construction of a Box-Behnken experimental design

The use of experimental designs is advantageous to ensure that predetermined responses are adhered to. A Box-Behnken experimental design (Minitab® V15, Minitab®, PA, USA) was therefore utilized for the generation of an optimal ovalbumin matrix for inclusion into the delayed-release system. Prior to the construction of Box-Behnken Experimental Design, the three independent variables highlighted were stressed to their maximum and minimum limits. This was undertaken by increasing and decreasing the respective variable concentrations and ascertaining their relevance as design parameters. For this, primarily *in vitro* probiotic release analyses were conducted. Whilst the formulation considerations are important, the therapeutic need to deliver the correct number of viable bacteria and ensure their survival during formulation processes was vital. Each probiotic release profile was therefore analyzed and the design parameters chosen. Formulation concerns were also taken into consideration in choosing the parameters.

4.2.4 Determination of the upper and lower independent limit parameters of ovalbumin concentration, genipin concentration and pH value

The upper and lower independent limits of ovalbumin concentration was determined through the addition of probiotic powder (10mg) to 1.5mL of raw egg ovalbumin and formulating ovalbumin granules as stated in Chapter 4, Section 4.2.2. Phosphate Buffer Saline (PBS) at pH 7.0 (the pH value utilized in the preformulation studies) and 0.01%v/v genipin solution were used as the denaturing and crosslinking solutions respectively. The procedure was repeated using varying ovalbumin concentrations from 0.2 to 2.0mL.

The upper and lower independent limits of genipin concentration to be included in the experimental design were determined utilizing the granulation method stated in Section 4.2.2 with genipin concentrations varying from 0.0001% to 0.1%v/v (ovalbumin and pH value remained constant). For the determination of the upper and lower limits of PBS pH value to be included in the experimental design, the granulation method stated was repeated using varying pH values between 1.2 and 12.0 (ovalbumin and genipin concentration remained constant). The upper and lower limit for each independent variable was determined through *in vitro* probiotic release analysis as stated in Chapter 3, Section 3.2.6.7.

4.2.4.1 *Fourier Transform Infrared Spectroscopy of the ovalbumin system crosslinked with genipin*

Fourier transform infrared (FTIR) spectroscopy was carried out on the freeze-dried crosslinked and non-crosslinked ovalbumin systems using a Perkin Elmer Spectrum 2000 FTIR spectrometer with a MIRTGS detector (PerkinElmer Spectrum 100, Llantrisant, Wales, UK). The structural properties of the crosslinked and non-crosslinked ovalbumin systems were thus determined. The respective samples were prepared as pellets against a blank background at a wave number ranging from 4000-650cm^{-1} with a resolution of 10. The spectrum software (Spectrum 100) was used for interpretation of the results.

4.2.5 Evaluation of formulations generated by the Box-Behnken design

Upon determination of the upper and lower limit for each independent variable, a formulation design was constructed utilizing the Box-Behnken experimental design. Each design formulation was prepared into granules and analyzed in triplicate. The generated design formulations are listed in Table 4.1.

Table 4.1: Formulations generated by the Box-Behnken experimental design.

Formulation	Std Order	Run Order	Pt Type	Blocks	Ovalbumin (mL)	Genipin (%v/v)	Buffer pH
1	14	1	0	1	0.875	0.0055	6
2	10	2	2	1	0.875	0.01	3
3	15	3	0	1	0.875	0.0055	6
4	7	4	2	1	0.25	0.0055	9
5	9	5	2	1	0.875	0.001	3
6	5	6	2	1	0.25	0.0055	3
7	4	7	2	1	1.500	0.01	6
8	8	8	2	1	1.500	0.0055	9
9	12	9	2	1	0.875	0.01	9
10	2	10	2	1	1.500	0.001	6
11	13	11	0	1	0.875	0.0055	6
12	3	12	2	1	0.250	0.01	6
13	1	13	2	1	0.250	0.001	6
14	11	14	2	1	0.875	0.001	9
15	6	15	2	1	1.500	0.0055	3

4.2.6 Determination of the Minimum Probiotic Concentration Time of each design formulation

The Minimum Probiotic Concentration Time (MPCT), the time taken for the number of viable bacteria required for functional health benefits to be delivered, was determined through *in vitro* probiotic release analysis of the design formulation. The MPCT was calculated through evaluation of the probiotic release profile and determining the time taken for the formulation to reach the required viable probiotic bacteria cell number of 10^8.

4.2.7 Determination of the flow properties of the design formulations

Flow properties are vital in processes involving granules due to the associated formulation reproducibility. Good flow properties ensure that the required amounts of granules are incorporated into each dosage form to ensure therapeutic effects for a patient. For the determination of the formulated granules flow properties, Carr's Compressibility Index (CCI)

and Angle of Repose (AoR) analyses were conducted using the method as outlined by Shah *et al*. (2008) with modifications.

The CCI of the respective formulations was determined by placing 0.5g of the granules formed into a 10mL measuring cylinder. The cylinder was tapped on a hard surface to simulate flow with the volume prior to and after tapping determined through use of a Vernier Caliper. The CCI was thereafter determined through calculation utilizing the Carr's Compressibility Index calculation noted in Equation 4.1.

$$Carr's\ Compressibility\ Index = \frac{(Tapped\ Density - Fluff\ Density)}{Tapped\ Density} \times 100 \qquad \text{Equation 4.1}$$

Where:
Fluff Density is the original height and
Tapped Density is the height after tapping.

The Angle of Repose of the respective formulation was determined by placing a glass funnel onto a retort 4cm above the surface of measurement. Granules (0.5g) were placed into the funnel and allowed to flow onto the measurement surface. The Angle of Repose was determined through calculation using Equation 4.2 by measuring the diameter and height of the resultant mound formed utilizing a Vernier Caliper.

$$Angle\ of\ Repose: Tan\phi = \frac{2h}{D} \qquad \text{Equation 4.2}$$

Where:
h is the granule height at centre point and
D is the diameter of the granule bed.

4.2.8 Determination of the matrix hardness of the design formulations

The matrix hardness of the granules was determined on the tablet slug prior to the dry granulation process using a calibrated Texture Analyzer as stated in Chapter 3, Section 3.2.6.3. A typical TA profile of matrix hardness of the ovalbumin slugs containing *L. acidophilus* is depicted in Figure 4.3. The indentation hardness was represented by conversion to the Brinell Hardness Number (BHN) (N/mm^2) calculated as per Equation 3.1.

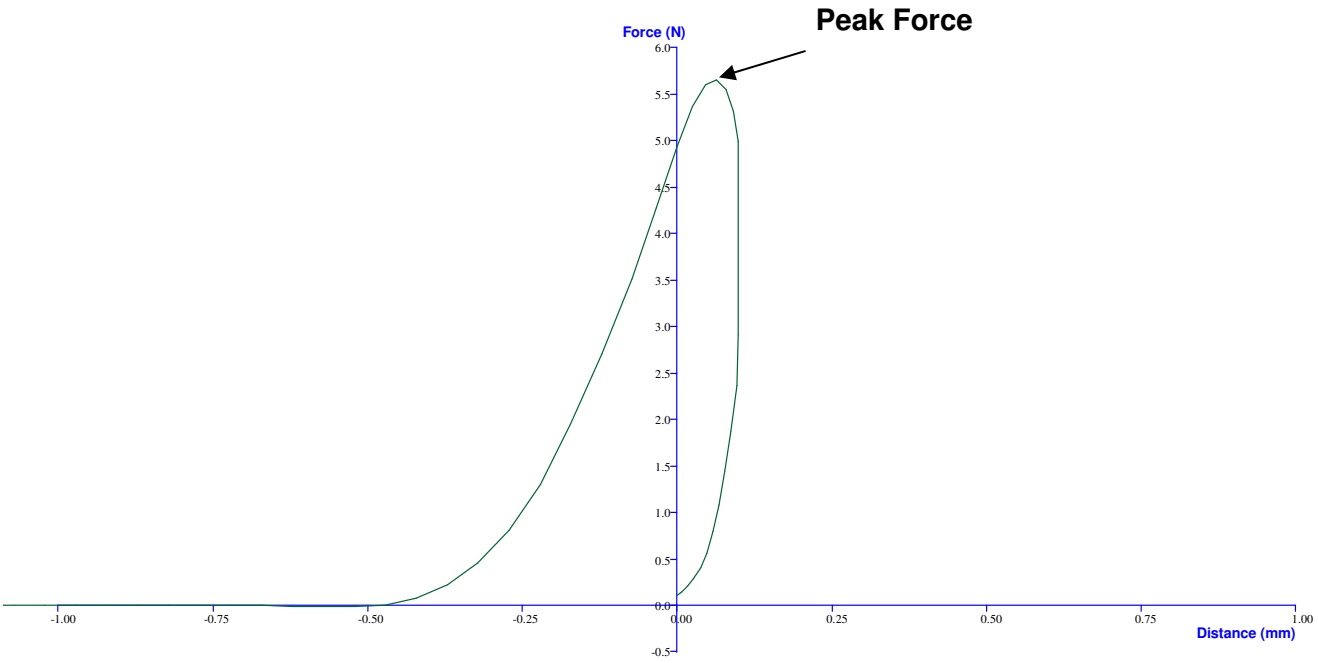


Figure 4.3: A typical TA profile generated for the matrix hardness determination of the ovalbumin slugs containing *L. acidophilus* (where hardness is the maximum height of the graph peak).

4.2.9 Statistical Analysis of the Box-Behnken experimental design

Statistical analysis of the conducted Box-Behnken experimental design was undertaken utilizing the Minitab® V15 software with Analysis of Variance (ANOVA) protocols.

4.2.10 Statistical optimization of the ovalbumin granules

Statistical optimization of the ovalbumin granules was undertaken utilizing Minitab® V15 software configured to give a MPCT of 4 hours and a minimum AoR and CCI depicting excellent flow properties. This would facilitate maximum probiotic release across the intestinal tract prior to the next dose of the antibiotic formulation (commonly taken 6-8 hours apart) in addition to excellent flow properties of the formulated granules.

4.2.11 Preparation of the optimized ovalbumin granule system

The optimized granule system was prepared as per the Box-Behnken experimental design optimization output utilizing 0.8868mL ovalbumin, 0.0058%v/v genipin and a pH buffer concentration of 6.9929. The optimized granules were formulated and underwent *in vitro* probiotic release analysis as per the methods previously described in Section 4.2.2 and Chapter 3, Section 3.2.6.7 respectively.

4.2.12 Determination of the Carr's Compressibility Index, Angle of Repose and Matrix hardness of the optimized granule system

The flow properties (CCI and AoR) and Matrix hardness of the optimized granule system were determined utilizing the methods outlined in Sections 4.2.7 and 4.2.8 respectively.

4.2.13 Static lattice atomistic simulations of the crosslinked ovalbumin-genipin complex

Molecular simulations were performed using commercial software HyperChem^{TM} 8.0.8 Molecular Modeling System (Hypercube Inc., Gainesville, Florida, USA) and ChemBio3D Ultra 11.0 (CambridgeSoft Corporation, Cambridge, UK). The structures of genipin (GNP) was built as a 3D model using ChemBio3D Ultra, while the structure of ovalbumin (Leu-Gln-Ala-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu) was generated using the built-in sequence editor module of HyperChem. The models were energy-minimized using a progressive-convergence-strategy where initially the MM+ force field was used followed by energy-minimization using the Amber 3 (Assisted Model Building and Energy Refinements) force field. The conformer having the lowest energy was utilized to create the ovalbumin-genipin complexes. A complex of one molecule with another was assembled by disposing the molecules in a parallel way and the same procedure of energy-minimization was repeated to generate the final models: OVA-GNP1, OVA-GNP5, and OVA-GNP10 corresponding to ovalbumin formulations with genipin crosslinking concentrations of 0.001%, 0.055, and 0.01%, respectively. Full geometry optimization was carried out in vacuum employing the Polak–Ribiere conjugate gradient algorithm until an RMS gradient of 0.001kcal/mol was reached. For molecular mechanics calculations in vacuum, the force fields were utilized with a distance-dependent dielectric constant scaled by a factor of 1. The 1-4 scale factors were electrostatic 0.5 and van der Waals 0.5 (Kumar *et al.*, 2011).

4.3 Results and Discussion

4.3.1 Independent variable determination for the conduction of the Box-Behnken experimental design

4.3.1.1 Ovalbumin concentration variable

To determine the upper and lower parameters of ovalbumin concentration, various concentrations of ovalbumin were analyzed with the addition of genipin and pH buffer at fixed solution concentrations. The upper value of the range of ovalbumin evaluated was determined by the highest ovalbumin concentration that could be accommodated in the hard gelatin capsule. This value was determined to be 2.0mL as concentrations higher that this value did not allow for the addition of further actives such as 250mg amoxicillin, the intended antibiotic in the Dual-Biotic System. The lowest ovalbumin concentration in the range tested was the minimum concentration required for a homogenous solution of probiotic bacteria to be formed in the formulation process. This value was determined to be 0.2mL as concentrations lower that this value resulted in slurry upon addition of the formulated *L. acidophilus* bacteria and failed to form a homogenous solution which was vital to allow for entrapment of the probiotic bacteria in the ovalbumin matrix prior to the addition of the pH buffer and the crosslinking genipin solution.

Results of the *in vitro* probiotic release analysis performed on the ovalbumin range described determined the upper and lower limits of ovalbumin that was to be utilized in the Box-Behnken experimental design. The lower limit of the ovalbumin variable was determined to be 0.25mL as this concentration caused a controlled release of probiotic bacteria, unseen in lower concentrations. The upper limit was determined to be 1.5mL as this concentration also displayed a controlled release and had a maximum viability after the analysis period within an acceptable range. Even though the MPCT was below an ideal value, the use of the moderately high genipin concentration warranted the inclusion of this concentration as the upper parameter of the ovalbumin concentration variable. The results of the *in vitro* probiotic release analysis for the determination of the upper and lower parameter of the ovalbumin concentration variable as described are shown in Figure 4.4.

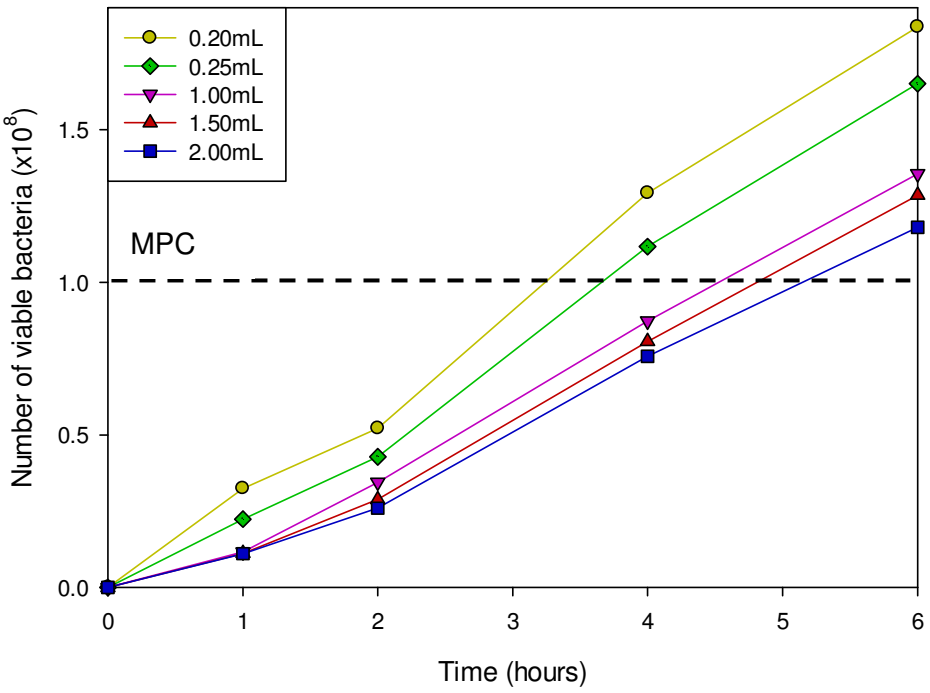


Figure 4.4: Probiotic release profiles of various concentrations of ovalbumin (MPC= Minimum Probiotic Concentration; n=3; SD≤ 0.42 in all cases).

4.3.1.2 Genipin concentration variable

The upper and lower parameters of genipin concentration were determined by varying the genipin concentrations between 0.0001% and 0.1% (v/v). All formulations were analyzed via *in vitro* probiotic release analysis. This analysis was conducted to determine the maximum viability attained after the tested time period of 6 hours as well as the manner of release. The upper value used in the range tested was the maximum concentration that could be added to the ovalbumin that did not result in the visual clumping of the protein structure. The amount of genipin solution added to the formulation was kept consistent to that of the amount of ovalbumin in the formulation allowing for an accurate correlation to be determined.

Results of this analysis showed that the ideal genipin concentration required for the delivery of *L. acidophilus* bacteria within a 4 hour time period laid within a concentration range of 0.001% to 0.01%v/v. At these concentrations, a controlled release of *L. acidophilus* was noted with the bacterial viabilities being at a higher level when compared to the 0.1%v/v genipin formulation (Figure 4.5). This meant that a lower release of *L. acidophilus* from the ovalbumin matrix was attained. Lower concentrations, such as 0.0001%v/v genipin concentration did show a higher viability after the tested time period, however, this can be attributed to the initial burst in bacterial concentration which was not determined at higher genipin concentrations. Controlled release was therefore not attained at a genipin

concentration of 0.0001%v/v. With regards to the formulations that showed a controlled release of *L. acidophilus*, the exponential increase in viabilities over time can attributed to the increase in metabolic activity associated with the growth enhancing effects of ovalbumin. The maximum viabilities of the probiotic bacteria remained relatively similar in the concentration levels ranging from 0.0001% to 0.01%v/v, even with the sudden burst seen in the 0.0001%v/v. This result can be attributed to the bacteria released being in the initial lag phase of bacterial growth (ranging from 0 to 3 hours as seen in Chapter 3, Section 3.3.2.1) in which the bacteria had not adapted to their environment to produce a greater increase in bacterial ATP levels as a result of bacterial replication.

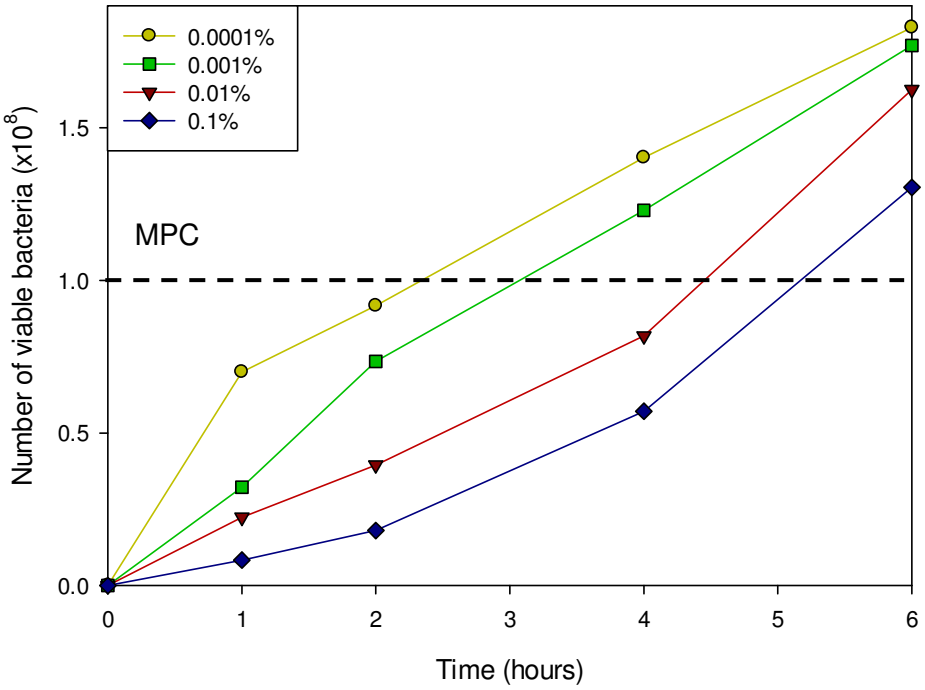


Figure 4.5: Probiotic release profiles of the various concentrations of genipin (n=3; SD≤ 0.022 in all cases).

4.3.1.3 pH value variable

The effect of buffers at various pH was chosen to be the third variable due to the positive results noted in the preformulation analyses conducted in Chapter 3. Proteins harbor an intrinsic buffering capacity which results in a controlled change in pH upon the addition of PBS. The effect of genipin as an additive on the pH can also not be ignored as the genipin solution itself has its own pH which would further alter the pH of the protein matrix in which the probiotic bacteria was entrapped. Various pH values ranging from 1.2 to pH 12.0 were therefore analyzed to determine the upper and lower parameters for this variable (Figure 4.6). The results determined similar viabilities at the required 4 hour MPCT data point. Minor

differences in the maximum viabilities were also recorded with pH 1.2 and pH 12.0 showing the lowest maximum viability after the analysis period and were therefore not considered as variable parameters. The effect of pH denaturation of the protein structure which resulted in erratic differences of the probiotic release profiles in Chapter 3 was not seen due to the high concentration of genipin included in this analysis. For the stated reasons, pH 3.0 and pH 9.0 was used as the lower and upper levels of the pH value variable, respectively.

A summarization of the process variables, the upper and lower parameters determined, as well as the formulation and viability considerations used in the determination of these parameters is listed in Table 4.2.

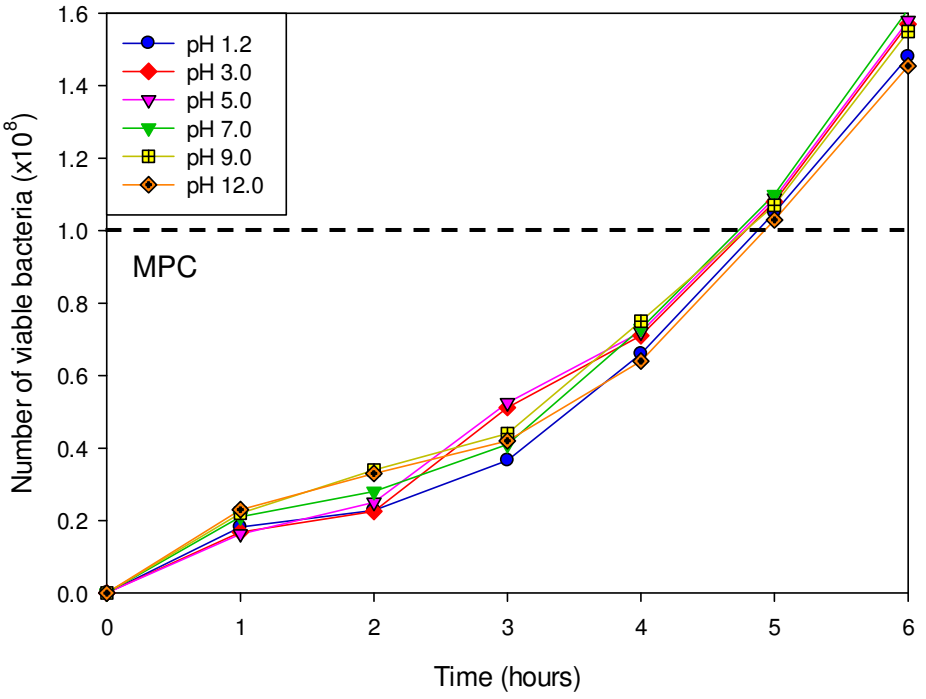


Figure 4.6: Probiotic release profiles of the various pH values of PBS (n=3; SD≤ 0.052 in all cases).

Table 4.2: Process variables determined with the upper and lower parameters utilized in the Box-Behnken experimental design.

Variable	Parameter	Reason for Parameter	
		Viability	**Formulation Consideration**
Ovalbumin concentration	Lower Parameter: 0.25	Lower concentrations in ovalbumin resulted in an initial burst in LA concentration.	Lower concentrations in ovalbumin resulted in separation of the probiotic bacteria from solution.
	Upper Parameter: 1.5	Higher concentrations of ovalbumin did not display the required viability after 4 hours.	Higher concentrations did not accommodate the capsule size.
Genipin concentration	Lower Parameter: 0.001%	Lower concentrations delivered bacteria at a faster, uncontrolled rate.	-
	Upper Parameter: 0.01%	Higher concentrations delivered fewer bacteria in the tested time period.	Higher concentrations showed visible saturation of the ovalbumin protein.
pH value	Lower Parameter: 3.0	Good viability. Lower pH values showed a decrease in viability after the tested time period.	-
	Upper Parameter: 9.0	Higher pH values showed a decrease in viability after the tested time period	-

4.3.2 Structural evaluation of the crosslinked ovalbumin system utilizing Fourier Transform Infrared Spectroscopy

FTIR spectra of the non-crosslinked ovalbumin as depicted in Figure 4.7a displays a large transmittance peak at 1634cm^{-1} which is attributed to C=O of the amide I band (primarily due to parallel β-sheets) related to the backbone confirmation of the protein structure (Aramwit *et al*., 2010). Evaluation of the transmittance peaks at 1633, 1642 and 1652cm^{-1} revealed approximately 37% parallel β-sheets, 33 random coils and 30% alpha-helices in the protein structure (Furlan *et al*., 2007). A characteristic peak at 1444cm^{-1} is due to the bending vibration of N-H molecules of the amide II band. Furthermore, a peak observed at 3282cm^{-1} can be attributed to O-H stretching vibrations, N-H extension vibrations and intermolecular H-bonds that occur in protein structures. Analysis of the crosslinked ovalbumin system revealed a significant increase in transmittance of the 1717cm^{-1} and 2153cm^{-1} bands when compared to the non-crosslinked ovalbumin matrix. This is due to the crosslinking of the free amide groups in the ovalbumin structure leading to decreased N-H stretching (Figure 4.7b). Other significant changes were determined at the 1281cm^{-1} band which is representative of a mixed vibration of CO-N and N-H (Wang *et al*., 2013). Decreases in transmittance were also observed for the 1517cm^{-1} and 1023cm^{-1} bands respectively. This was due to the decrease in the =C–H stretching peak with absorption peaks around 1370-1650cm^{-1} been shown to decrease upon the addition of genipin to proteins (Aramwit *et al*., 2010). Through these FTIR spectra, the crosslinking effect of genipin on ovalbumin was confirmed. This crosslinked matrix due to the decrease in free amide groups resulted in a more stable structure being formed ultimately controlling the release of *L. acidophilus* upon exposure to probiotic release media.

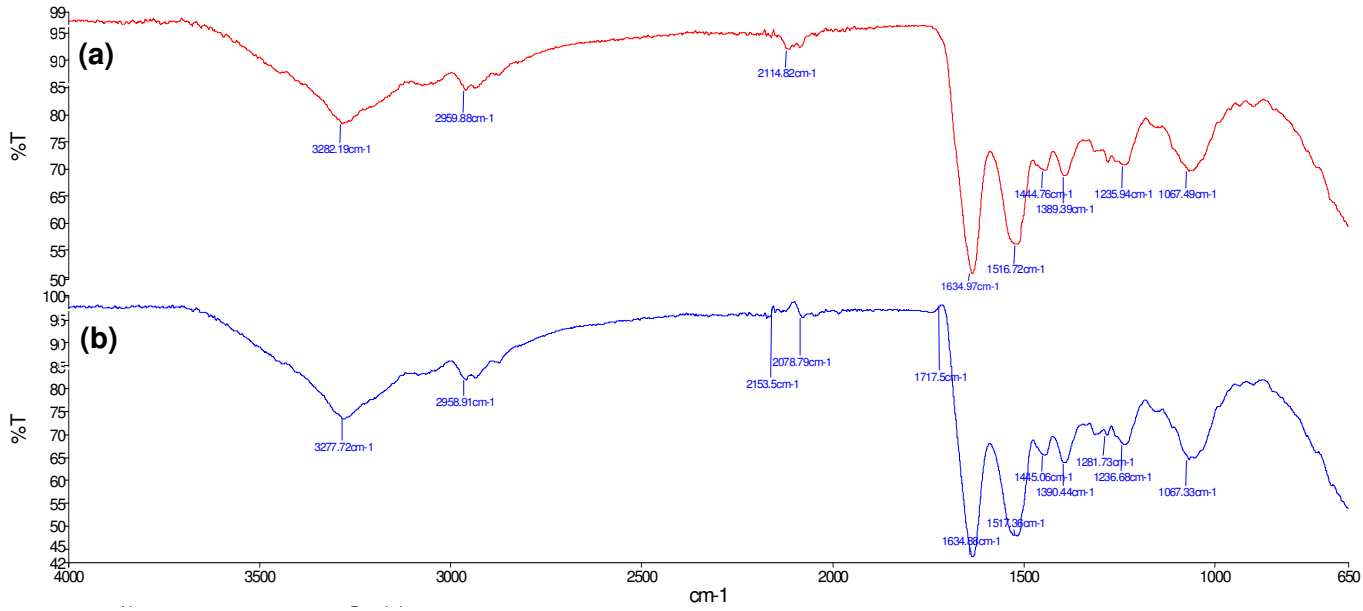


Figure 4.7: FTIR Spectra of (a) the non-crosslinked and (b) crosslinked ovalbumin matrices.

4.3.3 Evaluation of the Box-Behnken experimental design responses

During the formulation and evaluation of each design formulation, various responses were determined. These included MPCT (required for the therapeutic effects) as well as the AoR and CCI (measurements of granule flow properties). The hardness of the tablet slugs prior to granulation was also calculated to ascertain if the changes in probiotic release properties were as a result of the formulation changes or due to the difference in granule hardness. The MPCT, as discussed is the time required for the minimum viability of the probiotic bacteria to exert functional health benefits to a patient. For this analysis, this minimum viability was determined to be 3062.28cps.

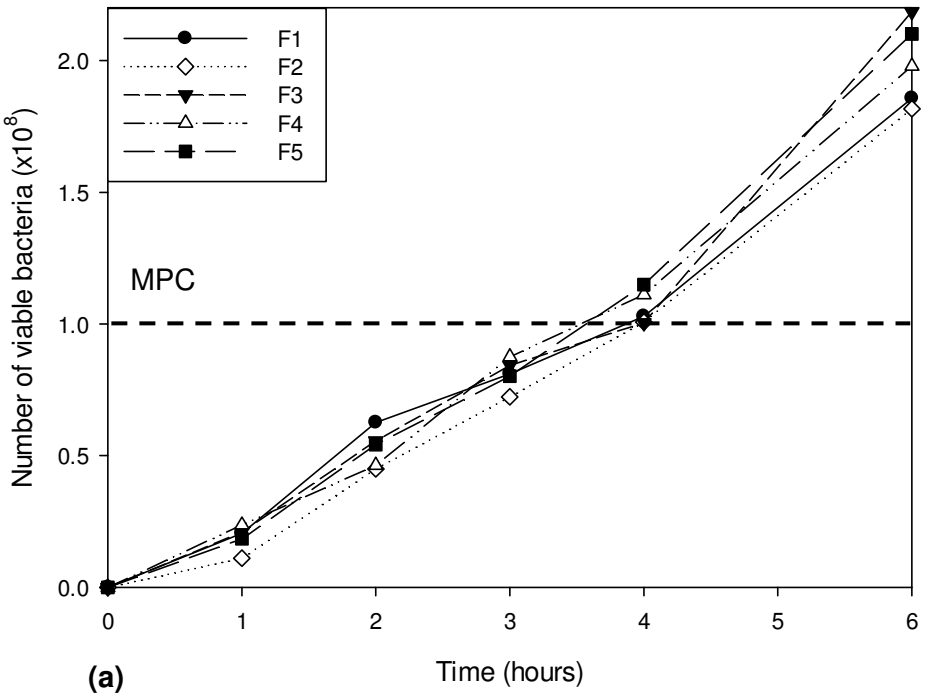
AoR and CCI are commonly used parameters for the determination of flow properties of a powder or a multi-particulate system such as granules (Shah *et al.*, 2008). This property is of significance during the formulation of any delivery system utilizing powders or granules to allow for efficient dosing of capsules or tablet dies. It is, however, of vital importance during the formulation of the Dual-Biotic System as this would ensure that the correct number of probiotic bacteria is incorporated into each capsule. A powder or granule system displays excellent flow properties at an AoR less than 20 and a CCI of less than 15 (Aulton, 2007). A full representation of these two analyses and the correlation to flow properties is listed in Table 4.3.

Table 4.3: Flow properties as determined by the CCI and AoR.

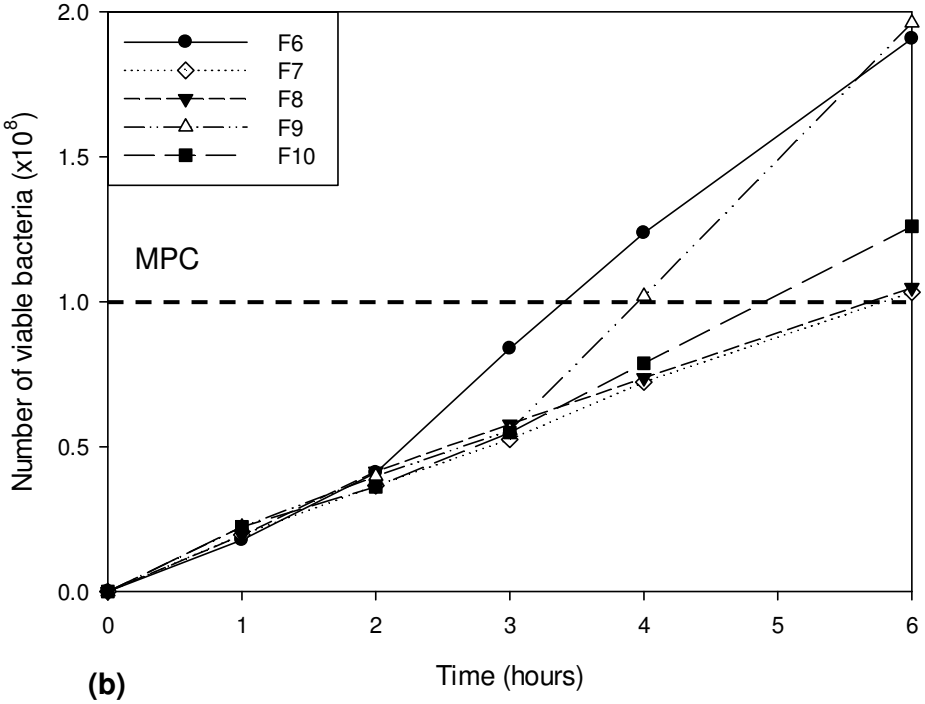
Carr's Compressibility Index (%)	Type of Flow	Angle of Repose (˚)
5-15	Excellent	<20
12-16	Good	20-30
18-21	Fair To Passable	30-34
23-35	Poor	
33-38	Very Poor	>35
>40	Extremely Poor	

4.3.3.1 Minimum Probiotic Concentration Time

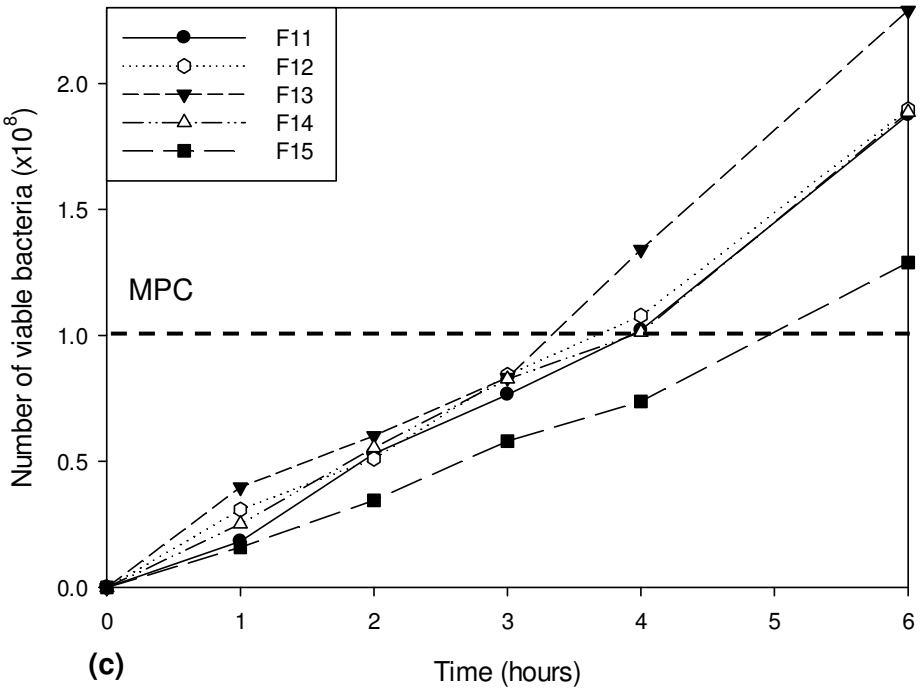
The probiotic release profiles of the respective design formulations as well as a comparative bar graph with regards to their MPCT are depicted in Figure 4.8. With a large difference in the MPCT, it was further determined that the ovalbumin variable had the greatest effect on MPCT (Figure 4.9a) with an increase in genipin concentration also resulting in a higher MPCT. This can be attributed to the greater crosslinking of the delivery system which resulted in a slower release over time and subsequent rise in MPCT values (Gander *et al*., 1989; Yan *et al*., 2012). This is noted in formulation 7, the highest MPCT, where the upper limit of ovalbumin and genipin concentration was utilized. A similar correlation was seen with an increase in pH value (Figure 4.9b), where a higher MPCT was noted with an increase in pH. This is due to the decreased viability of *L. acidophilus* at higher pH values resulting in longer period of time to reach the minimum ATP level required for functional health benefits and subsequent increase in MPCT (Sanchez *et al*., 2001). This correlation can be further identified in Figure 4.9c, where increases in genipin and pH resulted in higher MPCT values.


F1
F2
F3
F4
F5
MPC
Number of viable bacteria (x10^8)
Time (hours)
0.0
0.5
1.0
1.5
2.0
0
1
2
3
4
5
6

(a)


F6
F7
F8
F9
F10
MPC
Number of viable bacteria (x10^8)
Time (hours)
0.0
0.5
1.0
1.5
2.0
0
1
2
3
4
5
6

(b)



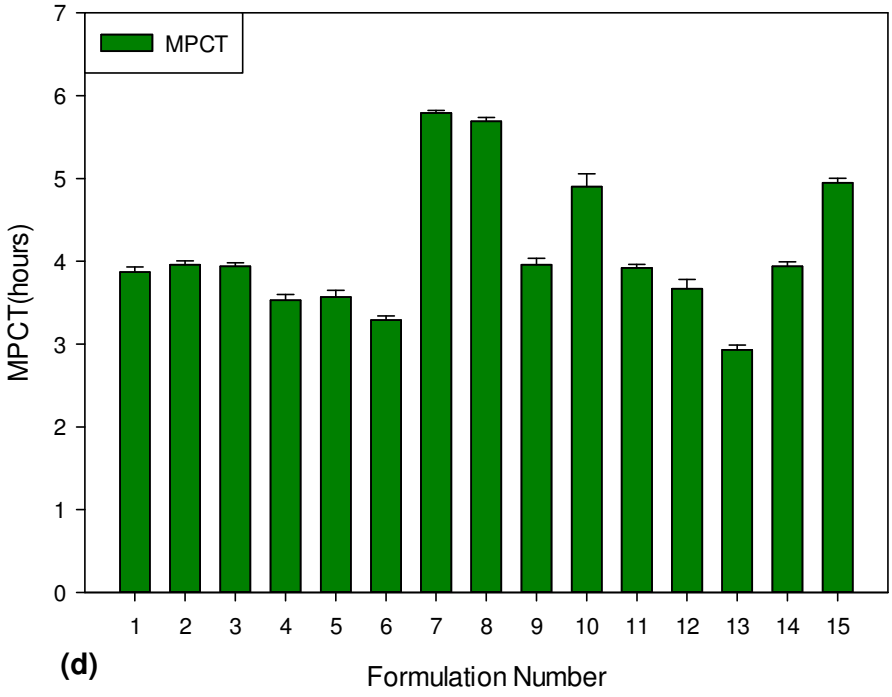


Figure 4.8: Probiotic release profiles of the respective Box-Behnken experimental design formulations (a-c; n=3; SD≤ 0.046 in all cases) and a summarization graph of MPCT values (d).

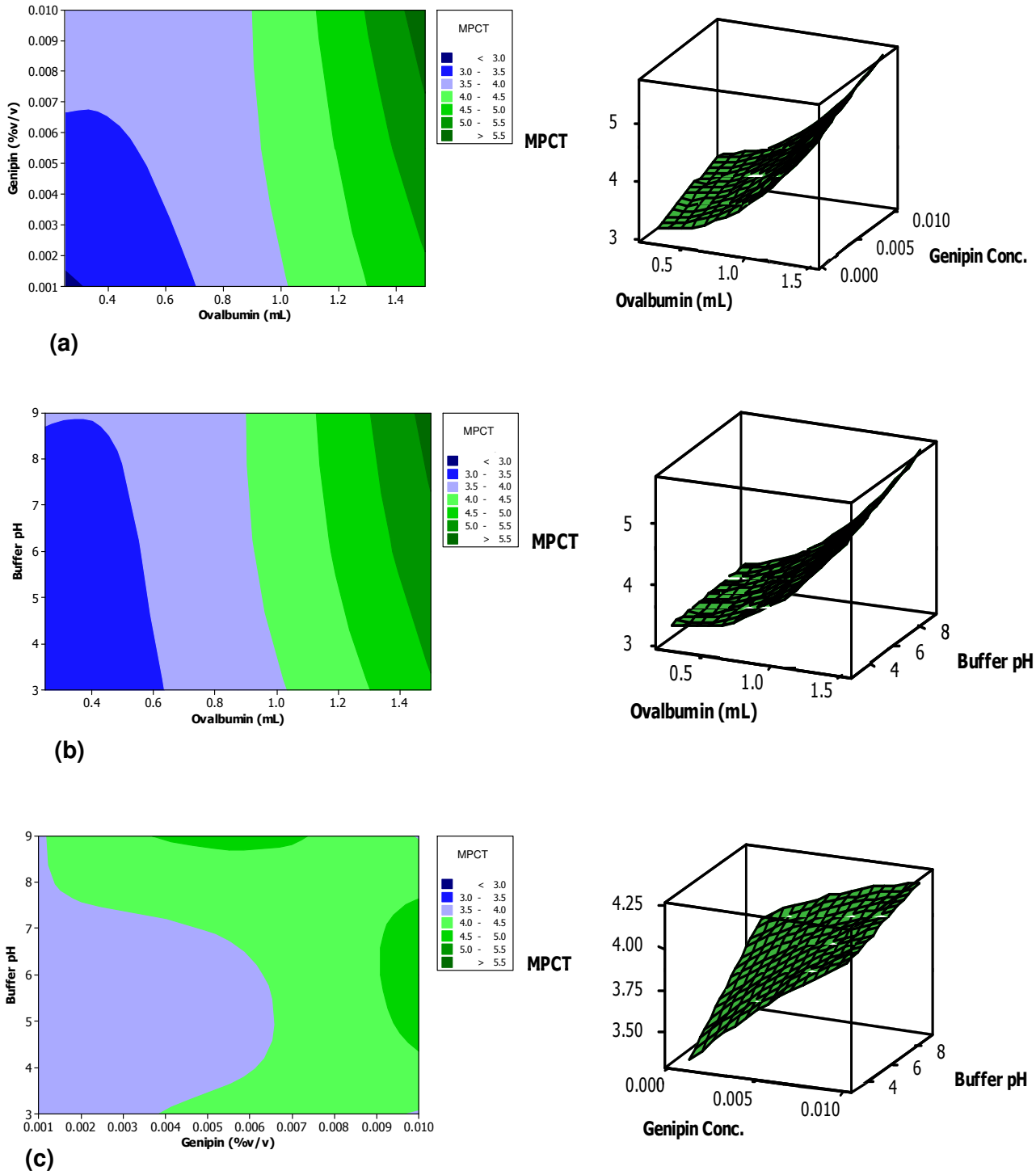


Figure 4.9: Contour and Surface Plots of the MPCT response.

4.3.3.2 Granule hardness

With the large differences in MPCT values noted, it was necessary to determine the matrix hardness of the granules prior *in vitro* probiotic release analyses. The result of these analyses would prove that the MPCT variations were due to ranging variable concentrations and that compression had no significant effect on the probiotic release. To determine the

matrix hardness of the slugs prior to granulation, texture analysis was employed. This would give a good estimation of the hardness of the granules prior to *in vitro* probiotic release analysis due to the dry granulation method employed being consistent in all formulations.

Results of the determination of the matrix hardness of the granules showed no significant differences in the BHN values of the tablet slugs with all values ranging between 3 to 4 (Figure 4.10). This therefore determined that the effect of compression did not have a significant effect on the probiotic release properties of the design formulations therefore removing the possibility of compression as an external influence.

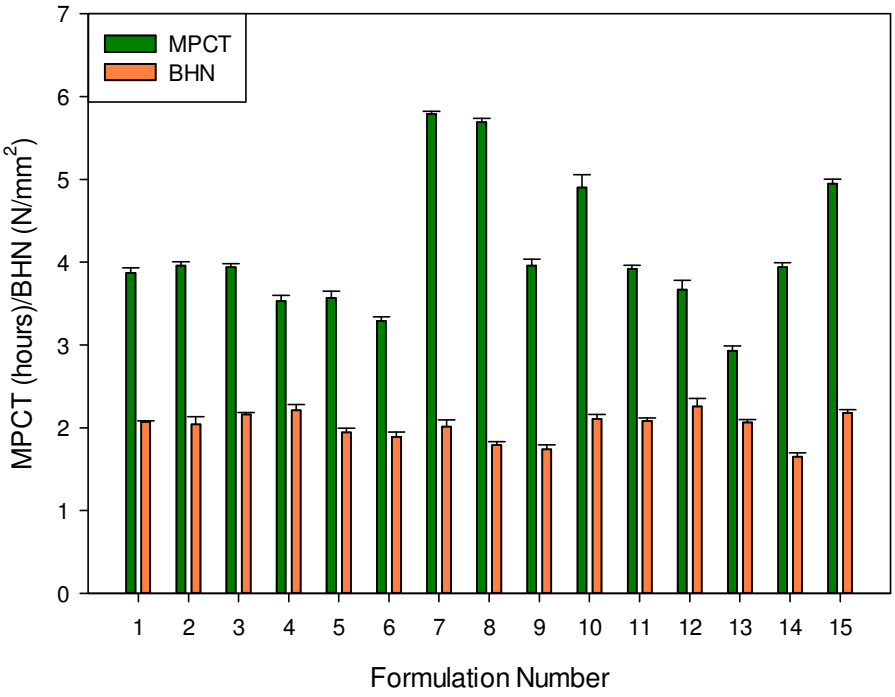


Figure 4.10: Comparison of MPCT and BHN of each Box-Behnken experimental design formulation.

4.3.3.3 Carr's Compressibility Index

CCI analysis determined varying values for each formulation (Figure 4.11). However, all these values were still maintained within the range of considered being excellent flow properties (5-15%). When the individual components of the delivery system were compared to each other, ovalbumin concentration had the greatest influence on CCI values (Figure 4.12a and 4.12b). It was determined that the lowest CCI values occurred at medium ovalbumin concentrations. This is noted in comparison of formulation 4 with 8, the lowest and highest CCI values, where the lower and upper limits of ovalbumin concentration respectively. This can be due to lower ovalbumin concentrations having a lower particle size

during the granulation process, resulting in greater inter-particular forces, thereby inhibiting granule flow. The higher ovalbumin concentration resulted in a greater particulate size, which upon the addition of force through tapping, resulted in decreased flow due to the granules being unable to flow unrestricted. When the effect of pH was compared, the resultant Carr's compressibility values showed that CCI values decreased with an increase in pH (Figure 4.12c).

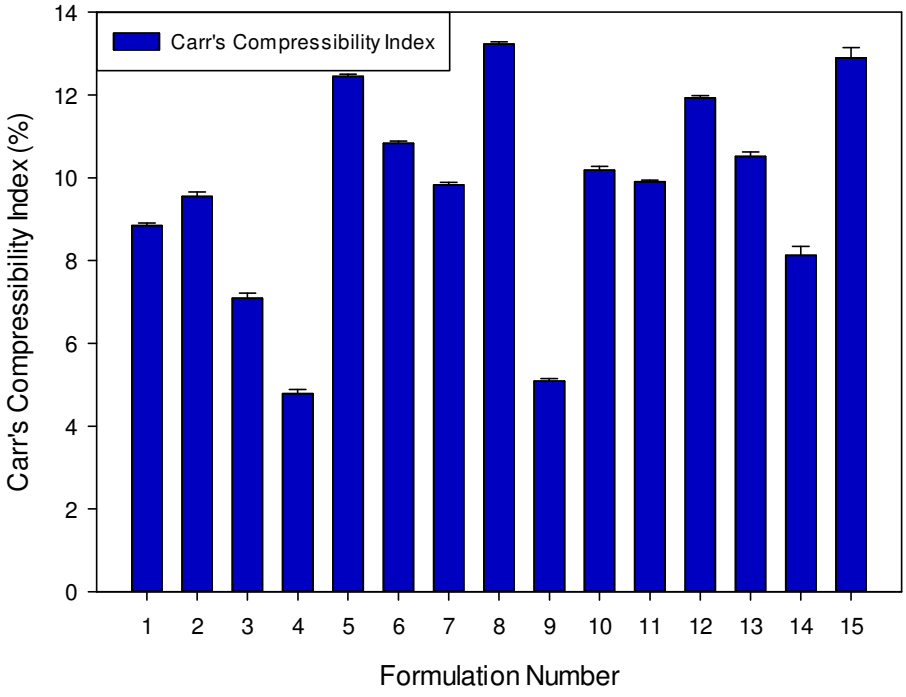


Figure 4.11: Bar graph depicting the CCI of the various design formulations.

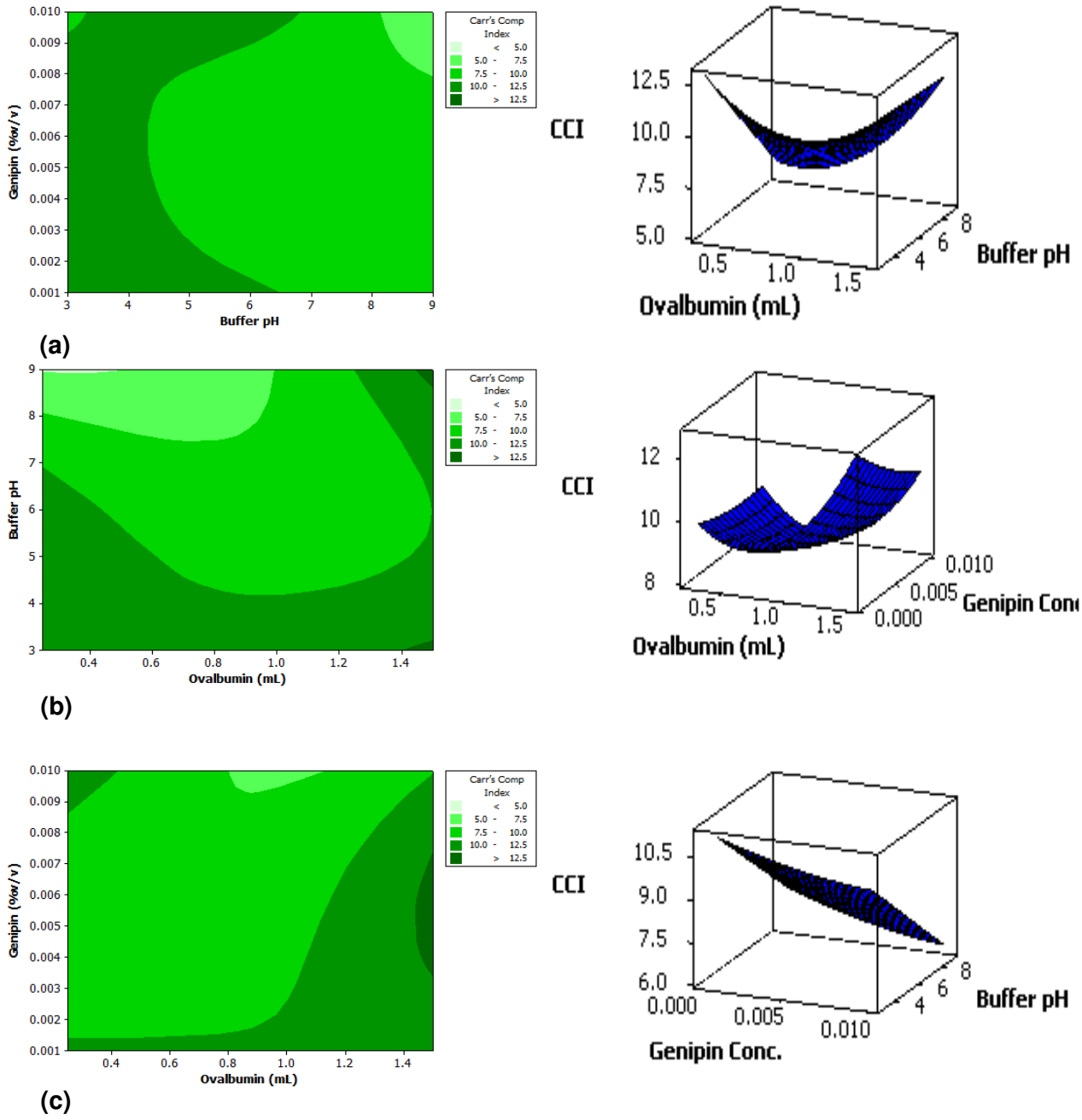


Figure 4.12: Contour and Surface Plots of the CCI response.

4.3.3.4 Angle of Repose

Angle of Repose was the second of the flow property analyses conducted. The premise behind the conduction of an Angle of repose analysis is that the higher the resultant mound formed upon passage through a funnel in relation to its diameter, the poorer the flow properties of the particle or powder. With regards to the ovalbumin granules formulated, the excellent flow properties of the granules resultant in a very low mound forming, often the size of a single granule. The results of this analysis like with the CCI analysis showed that all formulation showed excellent flow properties of below 20˚ (Figure 4.13). When the individual

components of the design formulations were compared, an increase in ovalbumin concentration resulted in a poorer flow as compared to the lower concentrations (Figure 4.14a and 4.14b). This can be observed where formulation 6 in comparison with formulation 7, where the lowest (6) and highest (7) AoR values were calculated from the lower and upper limits of ovalbumin concentration respectively. This can be attributed to the size of the granules formed. With regards to pH value and genipin concentration, no significant correlations were determined apart from higher and lower genipin concentrations showing poorer flow properties (Figure 4.14a and 4.14c).

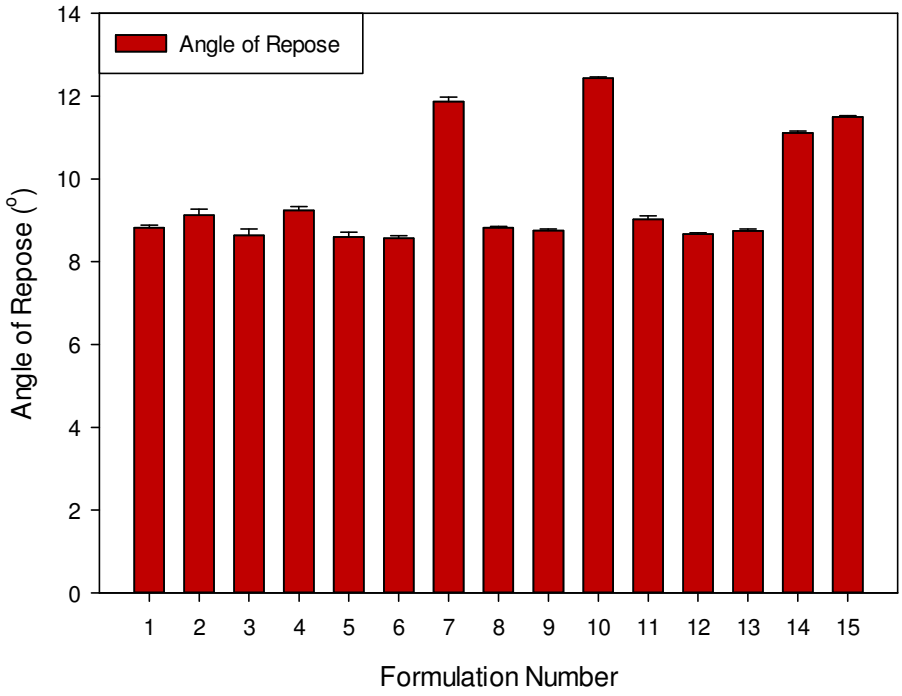


Figure 4.13: Bar graph depicting the AoR of the various design formulations.

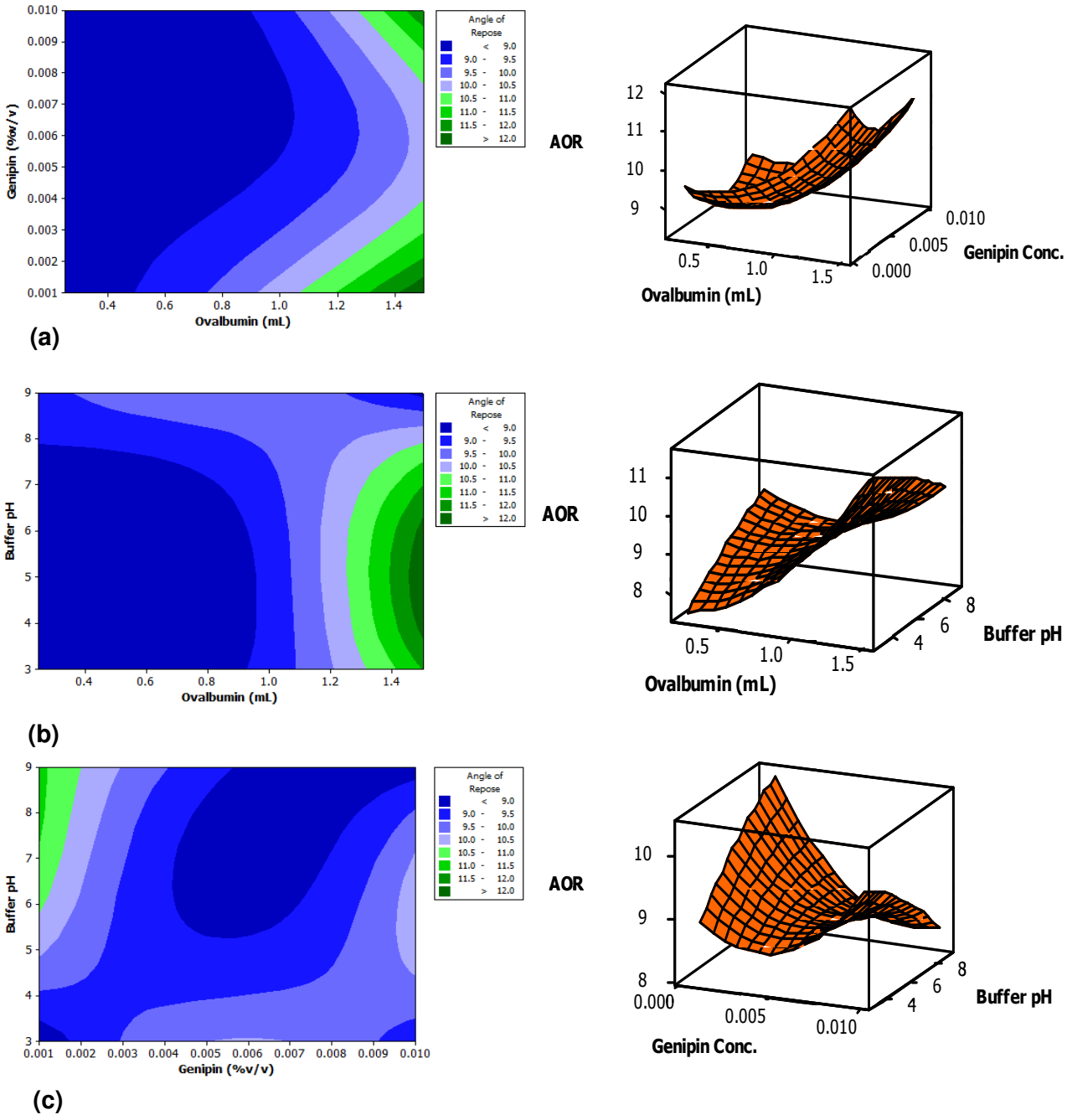


Figure 4.14: Contour and Surface Plots of the AoR response.

4.3.4 Statistical analysis of the Box-Behnken experimental design

4.3.4.1 Residual plots and Analysis of Variance

Residual plots (Figure 4.15) were utilized to determine the uniformity and distribution of data points determined for the experimental design responses. The normal probability plots of the residuals for all three design responses depict a uniform distribution of points along the straight line indicating a normal distribution of data unhindered by external influence. With regards to the residual versus fitted plots, random scattering of points on either side of 0

were observed indicating no trends with regards to the data points determined. Some clustering of scatter points was, however, seen with the AoR response. Evaluation of the histograms revealed a symmetrical distribution of the residuals. This property supports the probability of uniform distribution of data points. Residuals versus the order of the data plots indicated non-random error of the data set. The residuals are distributed randomly on either side of the 0 line indicating that no serial correlation can be determined.

A complete Analysis of Variance (ANOVA) was carried out on the input variables to ascertain which variable had a significant effect on the design responses (Table 4.4). A significant effect of any factor is indicated by a *p*-value ≤ 0.05 for that respective factor. This was, however, not determined with a majority of the factors, apart from the Ovalbumin^2 factor of MPCT, indicating that the factors had no significant effect with regards to the formulation outputs seen.

Table 4.4: A complete ANOVA analysis for the measured responses.

Term	***P* value**		
	MPCT	**CCI**	**AoR**
Constant	0.009	0.060	0.139
Ovalbumin	0.216	0.223	0.771
Genipin	0.261	0.939	0.794
Buffer pH	0.831	0.522	0.280
Ovalbumin^2	0.010	0.227	0.150
Genipin^2	0.697	0.895	0.211
Buffer pH^2	0.967	0.995	0.760
Ovalbumin* Genipin	0.748	0.741	0.816
Ovalbumin* Buffer pH	0.309	0.265	0.148
Genipin* Buffer pH	0.441	0.979	0.200

The complete regression equations generated for MPCT, CCI and AoR are indicated below in Equations 4.3, 4.4 and 4.5 respectively:

MPCT = 2.88 - 0.95 [Ovalbumin] + 111.91 [Genipin] + 0.04 [Buffer pH] + 1.18 [Ovalbumin^2] - 2345.68 [Genipin^2] - 0.00 [Buffer pH^2] + 13.33 [Ovalbumin Genipin] + 0.07 [Ovalbumin* Buffer pH] - 6.85 [Genipin* Buffer pH]*

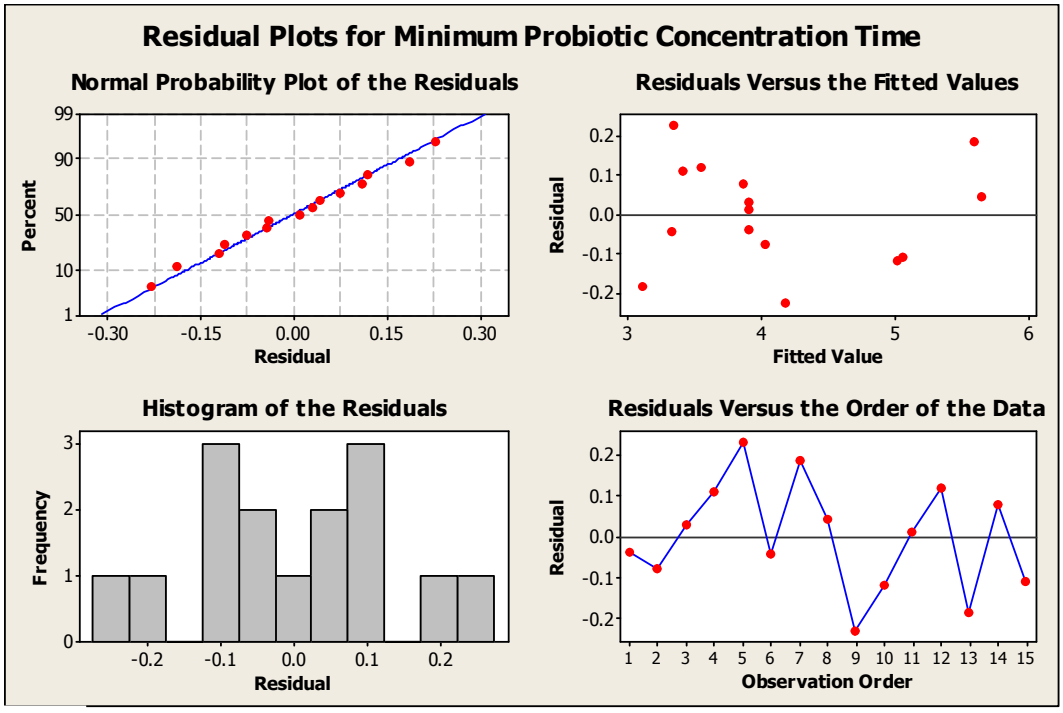
Equation 4.3

CCI = 19.06 - 10.77 [Ovalbumin] - 81.64 [Genipin] - 1.35 [Buffer pH] + 4.66 [Ovalbumin^2] + 9067.90 [Genipin^2] + 0.00 [Buffer pH^2] - 157.63 [Ovalbumin Genipin] + 0.85 [Ovalbumin* Buffer pH] - 2.59 [Genipin* Buffer pH]*

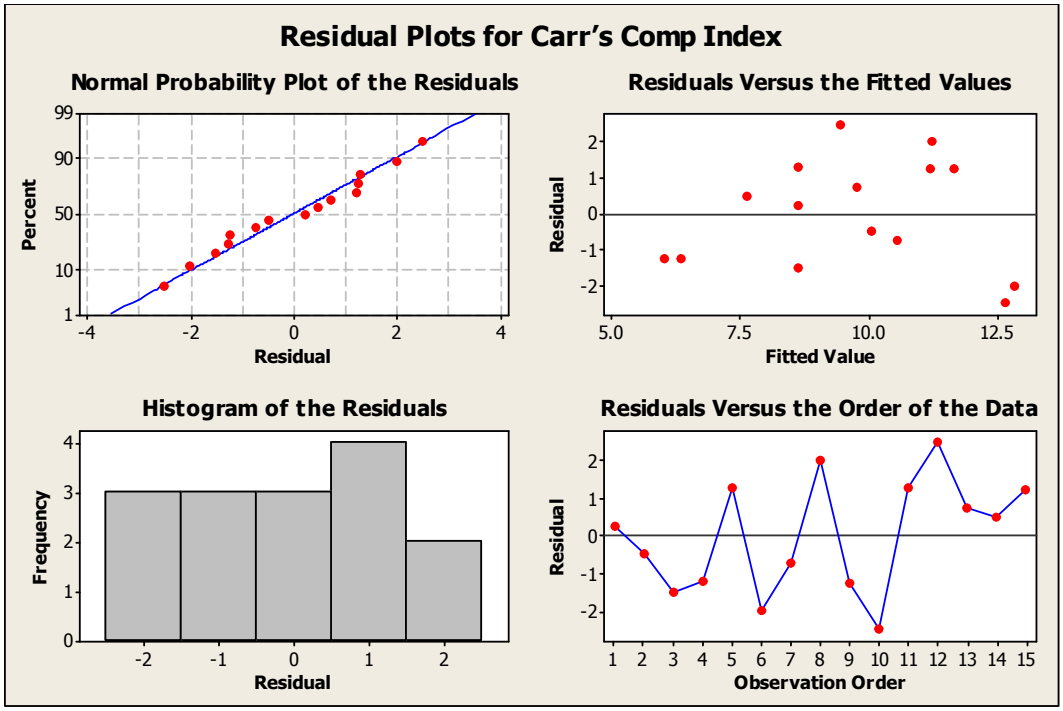
Equation 4.4

AoR= 5.3 + 0.9 [Ovalbumin] – 107.7 [Genipin] + 0.9 [Buffer pH] + 2.2 [Ovalbumin^2] + 36117.3 [Genipin^2] - 0.0 [Buffer pH^2] - 42.7 [Ovalbumin Genipin] - 0.4 [Ovalbumin* Buffer pH] - 53.5 [Genipin* Buffer pH]*

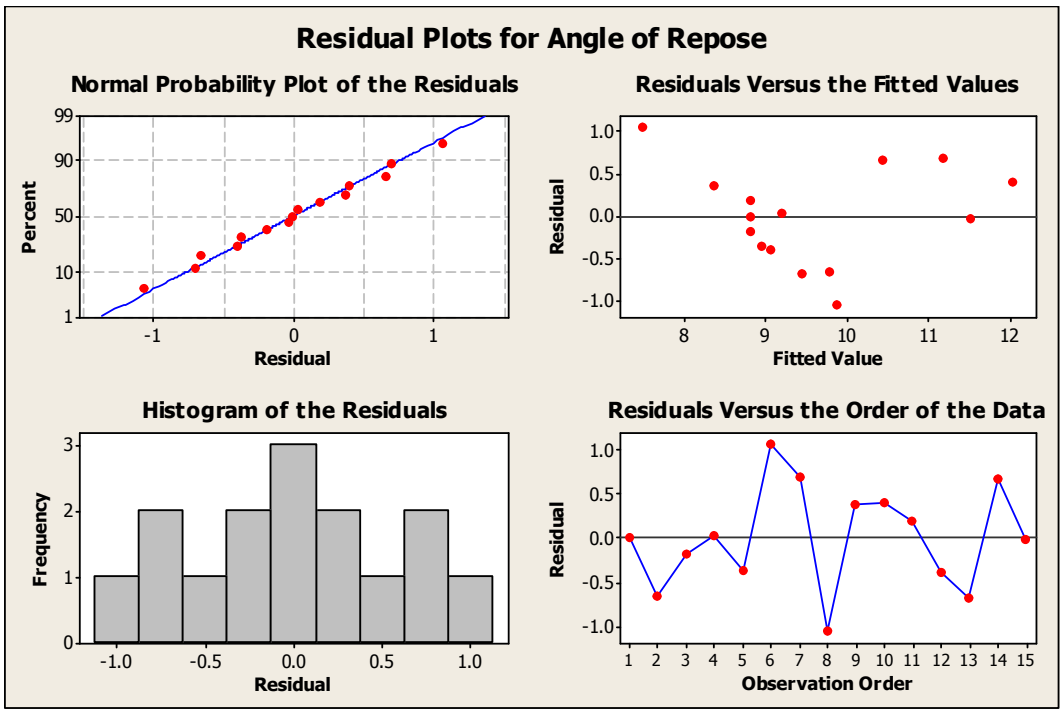
Equation 4.5



(a)



(b)



(c)

Figure 4.15: Residual plots of the Box-Behnken experimental design responses (a) MPCT, (b) CCI and (c) AoR.

4.3.4.2 Main Effect and Interaction plots

Evaluation of the effects of formulation input variables on design responses through the use of Main Effect Plots was advantageous in ascertaining a correlation between variable changes and responses seen. Interaction plots were also of use in determining the degree of variable interaction with respect to the various design responses. These plots were processed for the three design responses, MPCT, CCI and AoR, by comparing the individual input variables against the mean response values seen and the interactions thus determined. In each instance, the interaction plot that is provided displays the greatest intersection of input variable concentrations. This was conducted by alternating the independent and dependent graph axis variables and the interaction plot chosen.

MPCT Main Effect and Interaction plots

Evaluation of the MPCT Main effect plot revealed that the ovalbumin concentration had the largest effect on MPCT, with an increase in ovalbumin concentration resulting in a higher MPCT value (Figure 4.16). This can be due to the larger ovalbumin matrices releasing *L. acidophilus* at a slower rate resulting in a higher MPCT. The effect of genipin and pH buffer concentrations is also of importance as an increase in both variables results in a greater MPCT value. This is due to the increased crosslinking of the ovalbumin matrix resulting in higher MPCT values. The MPCT Main effect plot provides a good correlation with the surface and contour plots and ANOVA results provided, with more emphasis with respect to the effect of the individual input variable concentrations on the design responses.

Evaluation of the interaction plots for MPCT revealed the disordinal (non-parallel) interaction of the input design variables. This result therefore confirms that the interaction of variables was influential in the determination of MPCT values.

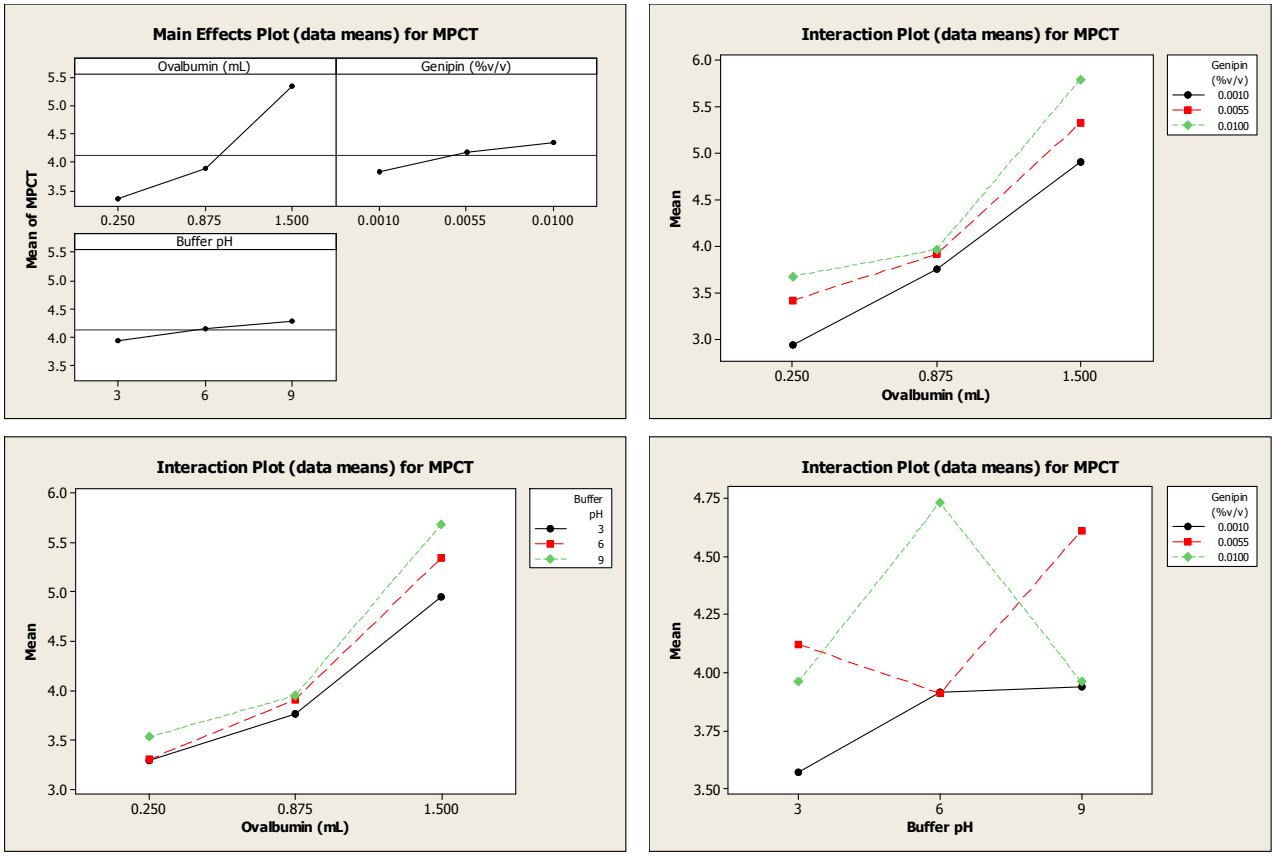


Figure 4.16: Main Effect and Interaction plots for the MPCT response.

CCI Main Effect and Interaction plots

Evaluation of the CCI Main effect plot revealed that the ovalbumin concentration had the largest effect on CCI values, with lower and higher ovalbumin concentration resulting in higher CCI values (Figure 4.17). This is due to smaller particles having greater inter-particular forces, as stated in Section 4.3.3.3, resulting in less flow, with the large particles unable to flow as easily as the medium sized granules. An interesting correlation was, however, determined between genipin concentration and CCI. Results of this analysis revealed that an increase in genipin concentration resulted in better flow. This could be due to the more rigid ovalbumin structure forming more stable granules that were able to flow easier. The effect of pH buffer was also noted with higher concentrations resulting in better flow properties.

Evaluation of the interaction plots for CCI also revealed the disordinal (non-parallel) interaction of the input design variables. This result, however, was more considerable than that seen with MPCT, with more interactions determined. This therefore confirms that the interaction of variables was highly influential in the determination of CCI values.

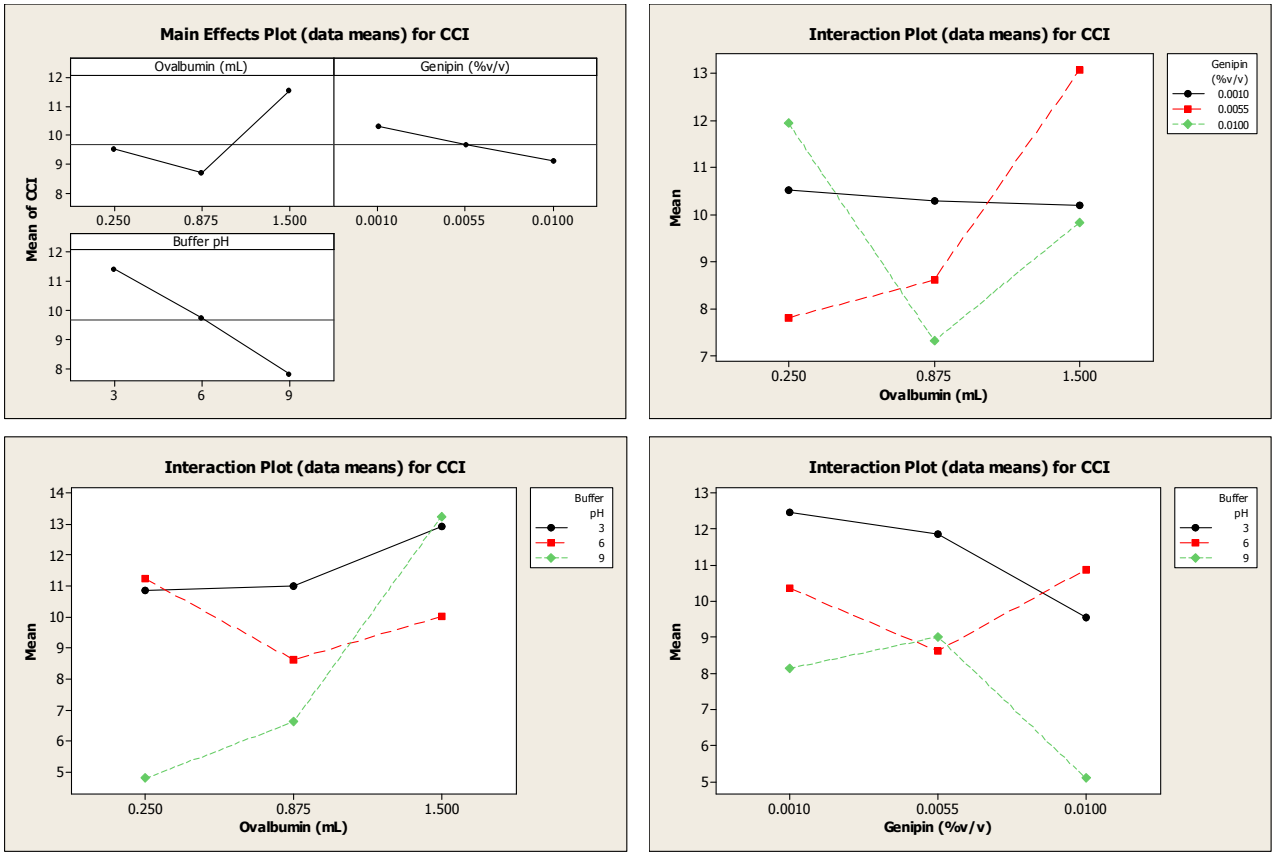


Figure 4.17: Main Effect and Interaction plots for the CCI response.

AoR Main Effect and Interaction plots

Evaluation of the AoR Main effect plot also revealed that the ovalbumin concentration had the largest effect on AoR values, with higher ovalbumin concentration resulting in higher CCI values (Figure 4.18). This result was, however, different to the CCI effects of lower ovalbumin concentrations. This can be attributed to the AoR analysis itself. The AoR analysis allows for movement of particles after impact on the tablet surface. As previously stated, the resultant mound formed was often the size of a single granule, which in lower ovalbumin concentrations resulted in much smaller granules sizes and therefore lower AoR values. This analysis also revealed that an increase in genipin concentration resulted in better flow (9.0-9.5 mean AoR). This further correlates with trend seen in the CCI determination of the effect of genipin concentration. The effect of pH on flow was not as considerable as with CCI with AoR values remaining constant throughout the pH range. This can be further attributed to the granule size effecting AoR values.

Evaluation of the interaction plots for AoR further revealed the disordinal (non-parallel) interaction of the input design variables with respect to granule flow. This result, however,

was important with a large number of interactions determined. This therefore confirmed that the interaction of variables was highly influential in the determination of AoR values.

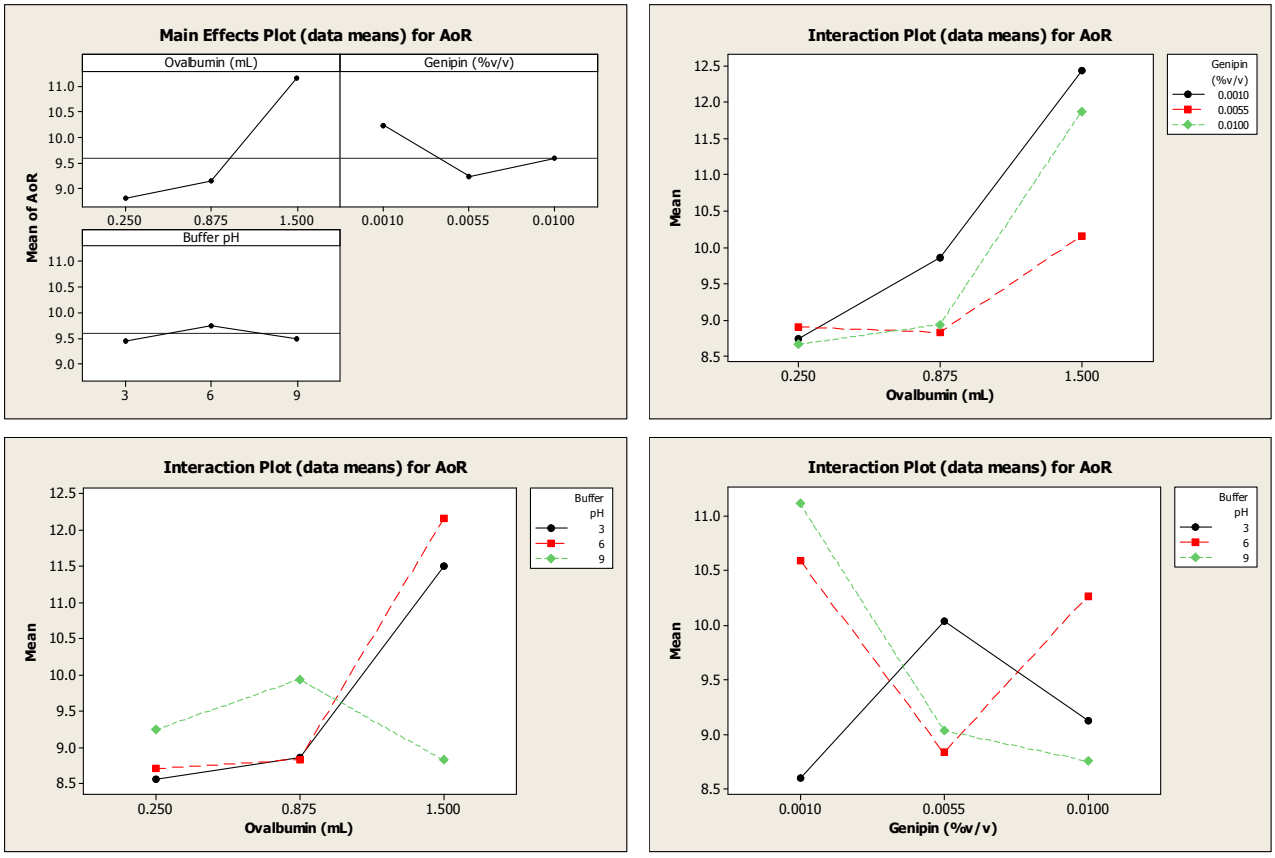


Figure 4.18: Main Effect and Interaction plots for the AoR response.

4.3.5 Response optimization of the ovalbumin granules

Upon completion of the Box-Behnken experimental design, statistical optimization was undertaken utilizing the responses determined during the formulation and analysis of the 15 design formulations. The parameters of the optimization were the targeting of the delivery system to have an MPCT of 4 hours and a minimum CCI and AoR. A summary of the parameters used in the statistical optimization are listed in Table 4.5 with the optimization protocol employed in the determination of the ideal methodology for the parameters determined depicted in Figure 4.19. The desirability index in the optimization of the crosslinked ovalbumin granules was determined to be 1.0000, the ideal value that can be attained.

Table 4.5: Response optimization parameters for the Box-Behnken experimental design.

Formulation Response	Optimization Parameter
Minimum Probiotic Concentration Time (MPCT)	Target: 4 hours
Carr's Compressibility Index (CCI)	Minimize
Angle of Repose (AoR)	Minimize

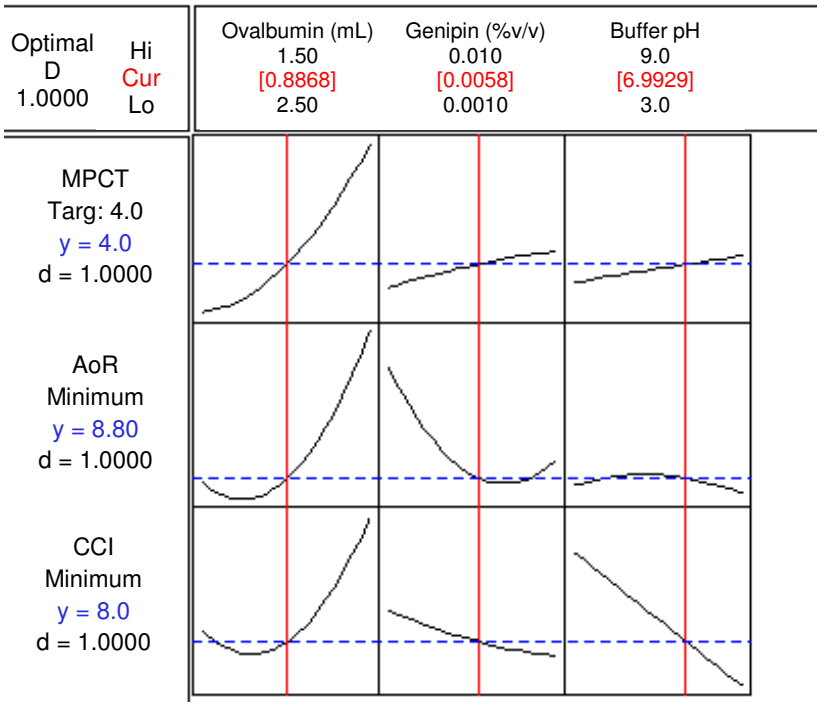


Figure 4.19: The optimization protocol employed in the determination of a MPCT of 4 and a minimum CCI and AoR. A desirability of 1.0000 was attained.

4.3.6 Experimental responses of the optimized system

With the optimization protocol utilized and the ideal methodology for a MPCT of 4 hours as well as a minimal AoR and CCI determined, it was necessary to evaluate the accuracy of the optimized methodology and determined its desirability to predicted values. The experimental design responses for the optimized system determined a MPCT of 3.98 hours (Figure 4.20) and AoR and CCI results similar or within the lower range of the responses predicted by the Box-Behnken experimental design (8.93 and 6.74 respectively). The Matrix hardness of the optimized formulation was also evaluated with a BHN value of 2.09 determined. This was

also within the range determined throughout the conduction of the Box-Behnken experimental design.

Evaluation of the design responses revealed a close agreement between the respective experimental and predicted values (Table 4.6). The close correlation of MPCT (99.50%), AoR (98.54%) and CCI (84.25%) revealed the statistical significance of the optimized system and the success of the optimization protocol used.

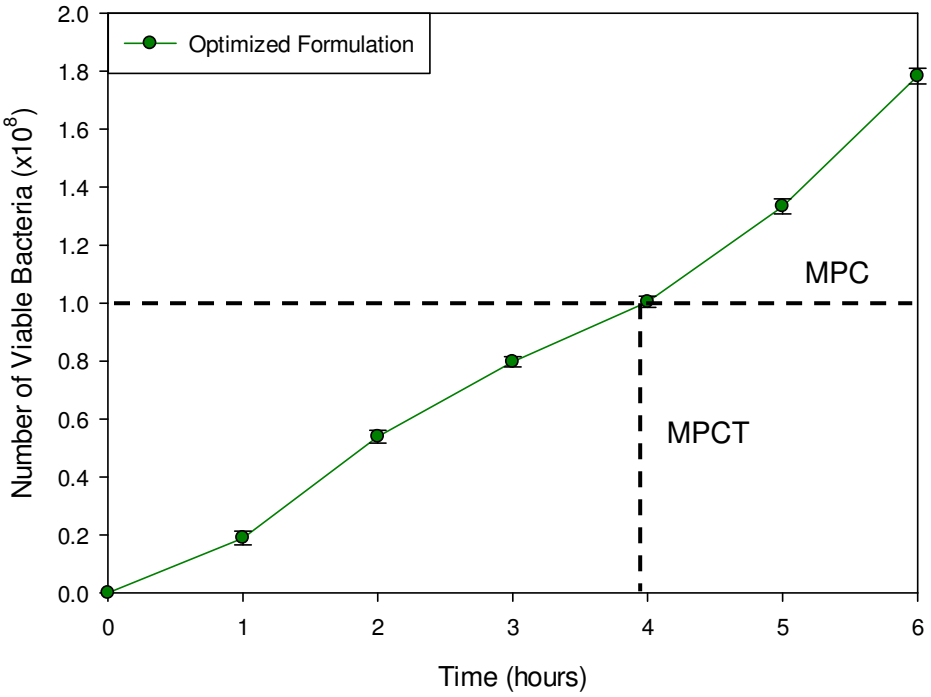


Figure 4.20: Probiotic release profile of the optimized granule system (n=3; SD≤0.027 in all cases).

Table 4.6: Predicted, experimental and desirability values of the optimized system.

Measured Response	Predicted Value	Actual Value	Desirability (%)
MPCT	4.00	3.98±0.03	99.50
CCI	8.00	6.74±0.08	84.25
AoR	8.80	8.93±0.11	98.54

4.3.7 Molecular mechanics assisted model building and energy refinements

Molecular mechanics energy relationship (MMER), a method for analytico-mathematical representation of potential energy surfaces, was used to provide information about the contributions of valence terms, noncovalent Coulombic terms, and noncovalent van der

Waals interactions for the protein/crosslinker interactions. The MMER model for potential energy factor in various molecular complexes can be written as:

$$\mathbf{E_{molecule/complex}} = V_{\sum} = V_b + V_\theta + V_\varphi + V_{ij} + V_{hb} + V_{el} \qquad \text{Equation 4.6}$$

Where, $V_{\sum}$ is related to total steric energy for an optimized structure, V_b corresponds to bond stretching contributions (reference values were assigned to all of a structure's bond lengths), V_θ denotes bond angle contributions (reference values were assigned to all of a structure's bond angles), V_φ represents torsional contribution arising from deviations from optimum dihedral angles, V_{ij} incorporates van der Waals interactions due to non-bonded interatomic distances, V_{hb} symbolizes hydrogen-bond energy function and V_{el} stands for electrostatic energy.

In addition, the total potential energy deviation, ΔE_{total}, was calculated as the difference between the total potential energy of the complex system and the sum of the potential energies of isolated individual molecules, as follows:

$$\mathbf{\Delta E_{Total(A/B)}} = E_{\mathbf{Total}(A/B)} - (E_{\mathbf{Total}(A)} + E_{\mathbf{Total}(B)}) \qquad \text{Equation 4.7}$$

The molecular stability can then be estimated by comparing the total potential energies of the isolated and complexed systems. If the total potential energy of complex is smaller than the sum of the potential energies of isolated individual molecules in the same conformation, the complexed form is more stable and its formation is favored (Yu *et al.*, 2008).

Energy-minimizations involving crosslinked-polymer morphologies

The energetic paradigms emerging out of the crosslinking of ovalbumin (OVA) by genipin (GNP) are given in Equations 4.8-12 and the corresponding energy minimized geometrical conformations are depicted in Figures 4.21 and 4.22 with Figure 4.23 representing the respective connolly molecular electrostatic surfaces. It is evident from Figure 4.22 that GNP crosslinked OVA molecule primarily via 1) secondary amide links –COC…NH and –C=O…NH, nucleophilic substitution reaction between the amine group of OVA and the ester group of genipin as explained by Butler and co-workers, (2003); 2) –OH…C=O hydrogen bonding; and 3) -OH…NH hydrogen bonding. The $\Delta E_{BINDING}$ = -17.175 kcal/mol in case of OVA-GNP1 proved that a stabilized complex was formed between OVA and GNP via all the three non-bonding interactions with electrostatic interactions playing the major part followed by van der Waals forces and hydrogen bonding (Eqs. 4.8-10). These stabilizing non-bonding

interactions confirmed the crosslinking mechanism of genipin over ovalbumin as explained in the FTIR characterization of the complex.

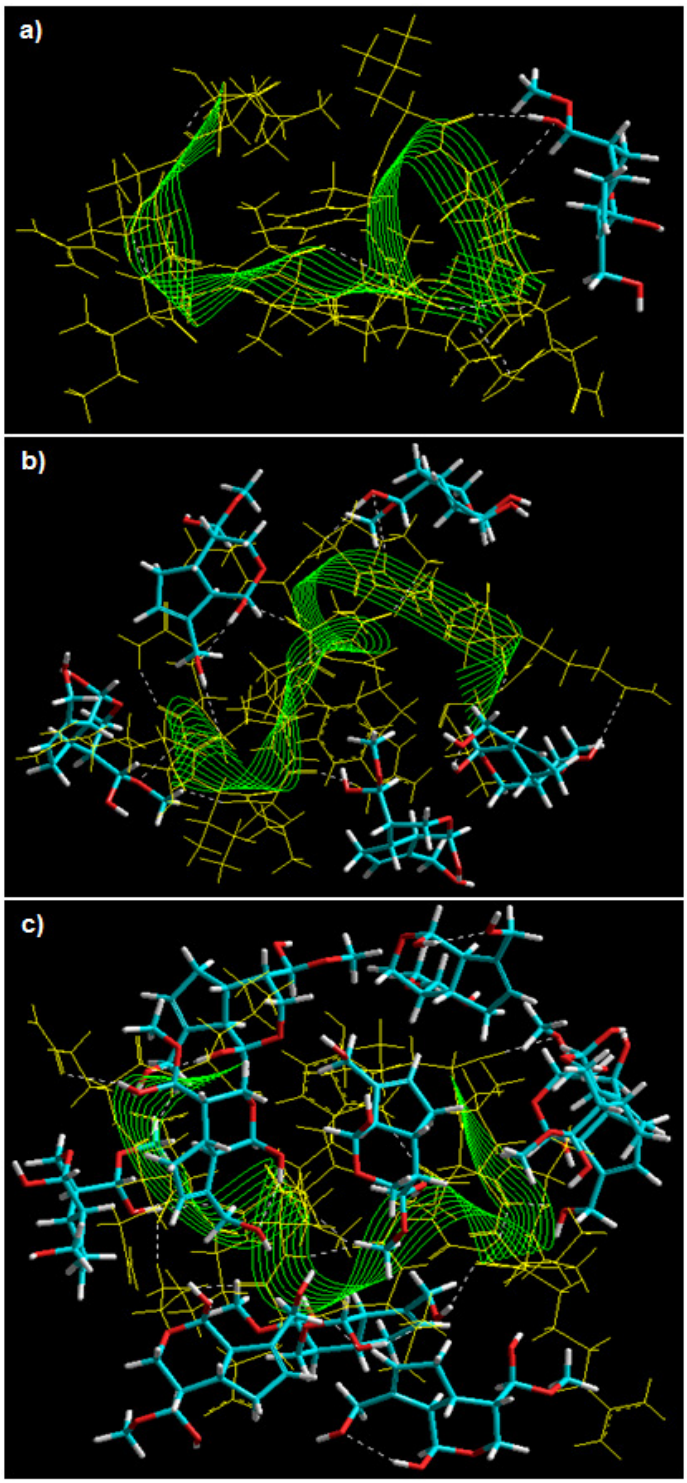


Figure 4.21: Visualization of geometrical preferences of ovalbumin (yellow stick rendering with green secondary ribbon structure) in complexation with (a) one genipin molecule (OVA-GNP1); (b) five genipin molecules (OVA-GNP5); and (c) ten genipin molecules (OVA-GNP10) after molecular mechanics simulations in vacuum. Color codes for genipin: C (cyan), O (red) and H (white).

$$\mathbf{E_{OVA}} = -276.821\,V_{\sum} = 4.001\,V_b + 20.569\,V_\theta + 29.478\,V_\varphi - 23.961\,V_{ij} - 6.478\,V_{hb} - 300.432\,V_{el} \quad (8)$$

$$\mathbf{E_{GNP}} = 23.004\,V_{\sum} = 0.500\,V_b + 19.591\,V_\theta + 2.647\,V_\varphi - 1.015\,V_{ij} - 0.751\,V_{hb} + 0.000\,V_{el} \quad (9)$$

$$\mathbf{E_{OVA\text{-}GNP(1)}} = -270.992\,V_{\sum} = 4.808\,V_b + 41.725\,V_\theta + 38.630\,V_\varphi - 33.886\,V_{ij} - 8.884\,V_{hb} - 313.385\,V_{el} \quad (10)$$

ΔE = -17.175kcal/mol

$$\mathbf{E_{OVA\text{-}GNP(5)}} = -234.064\,V_{\sum} = 6.437\,V_b + 114.011\,V_\theta + 47.602\,V_\varphi - 80.226\,V_{ij} - 11.903\,V_{hb} - 309.985\,V_{el} \quad (11)$$

ΔE = -72.263kcal/mol

$$\mathbf{E_{OVA\text{-}GNP(10)}} = -150.992\,V_{\sum} = 8.599\,V_b + 205.029\,V_\theta + 56.88\,V_\varphi - 119.748\,V_{ij} - 14.402\,V_{hb} - 287.352\,V_{el} \quad (12)$$

ΔE = -104.211kcal/mol

To elucidate the role of increasing the concentration of genipin for ovalbumin crosslinking, in line with the formulation groups, ovalbumin was modeled with 5 and 10 molecules of genipin by manual disposition of the crosslinker laterally along the 3D space of the protein, as shown in Figures 4.20b and 4.20c. After global energy and geometry minimization, the $\Delta E_{BINDING}$ values for the OVA-GNP5 was reported as -72.263 kcal/mol which was, understandably and exponentially, ~5 times more stabilized than the OVA-GNP1 molecular complex (Equations 4.8, 9, and 11). Four out of five genipin molecules successfully formed hydrogen bonds with the ovalbumin molecule forming a more robust and dense matrix as shown in Figure 4.21b and 4.23b. Interestingly, the hydrogen bonding and electrostatic energy components changed from -8.884 to -11.903kcal/mol (-ve energy of interaction ≈ stabilization of the complex) and -313.385 to -309.985kcal/mol (+ve energy of interaction ≈ destabilization of the complex), respectively, differing from the energy profiling of OVA-GNP1 where the electrostatic interaction played the major role. In case of OVA-GNP5; the van der Waals forces/dispersion forces primarily stabilized the crosslinking reaction and additionally were responsible for the torsional modifications inside the protein matrix leading to a denser matrix.

The final conformation model, OVA-GNP10, presented a $\Delta E_{BINDING}$ of -104.211 kcal/mol inflicted by the balanced destabilization and stabilization through the non-bonding electrostatic interactions and van der Waals forces, respectively (Equations 4.8, 9, and 12). Two definite conclusions can be drawn from these energy interactions:

1. The $\Delta E_{BINDING}$ in case of OVA-GNP10 was neither exponentially related to OVA-GNP1 nor to OVA-GNP5. Additionally, only 3 among the 10 molecules formed hydrogen bonding with the ovalbumin molecule. Furthermore, an intramolecular H-bonding was observed

within the GNP molecules. This represents an inherent saturation of the OVA functional groups leading to the maximum rigidification of the polymer matrix which may also be due to the “significant axial stress buildup” inside the polymer structure via the destabilization of even the bonding interactions such as the bond stretching and torsional contributions.

2. The total steric energy of the OVA-GNP10 (-150.992 kcal/mol) is almost half that of OVA alone (-276.821 kcal/mol) meaning that the OVA molecule in the former molecular complex is highly stabilized compared to the individual non-complexed OVA. This further confirms the solidification of the ovalbumin matrix and hence delayed release of the bioactive from the highly crosslinked polymer matrix in accordance with the experimental findings.

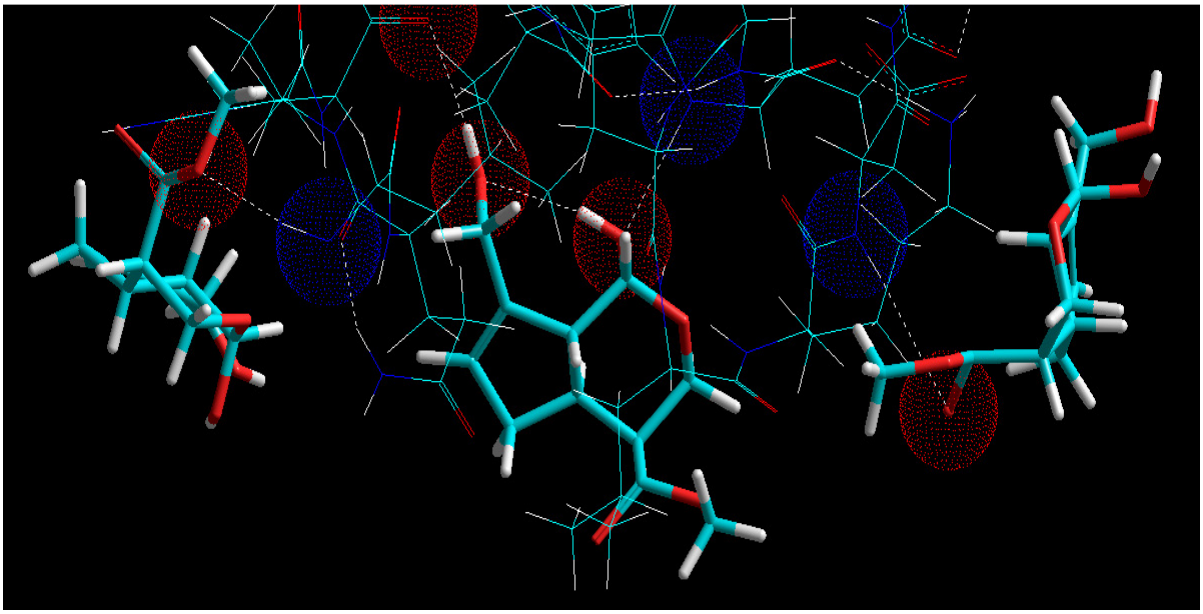


Figure 4.22: Visualization of functional interactions and preferences between ovalbumin (stick rendering) in complexation with the genipin molecules (tube rendering) after molecular mechanics simulations in vacuum. Color codes: C (cyan), O (red), N (blue) and H (white).

These molecular simulations are in corroboration with the observed experimental results where it was postulated that the immediate precipitation of the ovalbumin after the addition of maximum crosslinker concentration (0.01%) may be due to solidification and hence maximum saturation. Additionally, the spectral analysis corroborated with the mechanistic simulation in terms of functional groups involved in the crosslinking reaction (FTIR structural variation analysis) which further supported the *in silico-in vitro* correlation.

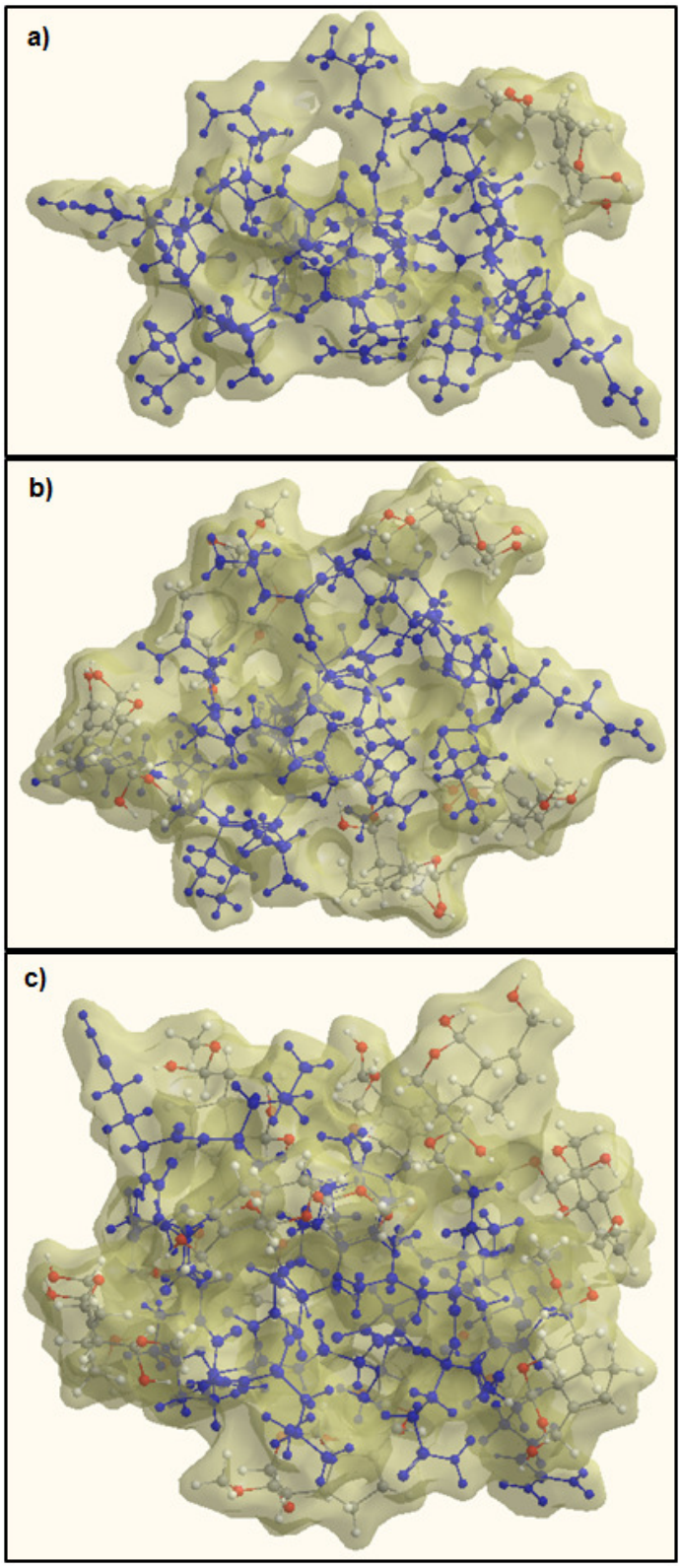


Figure 4.23: The connolly molecular electrostatic potential surfaces representing the matrix structure of ovalbumin (blue ball-and-stick rendering) in complexation with (a) one genipin molecule (OVA-GNP1); (b) five genipin molecules (OVA-GNP5); and (c) ten genipin molecules (OVA-GNP10) in transparent display mode. Color codes for genipin: C (grey), O (red) and H (white).

4.4 Concluding Remarks

The design, development and statistical optimization of the ovalbumin granules containing *L. acidophilus* were successfully undertaken in this chapter utilizing a 3-factor Box-Behnken experimental design. *In vitro* probiotic release analysis as well as formulation and therapeutic considerations were utilized for the determination of the independent design variables and their parameters. Statistical analysis and Analysis of Variance of the completed design determined the effect of the various independent variables on the set design responses chosen. Evaluation of the optimized granule system determined a close relationship between predicted and observed design responses, thereby validating the optimization protocol used. Molecular modeling was also performed on the crosslinked ovalbumin system detailing the molecular properties of the ovalbumin matrix with a distinct correlation between the design results and molecular modeling noted. The optimized ovalbumin granules were characterized in the next chapter in addition to the incorporation of the optimized granules into a delayed release system. The inclusion of the delayed release granules into the Dual-Biotic System was also discussed with the Dual-Biotic System formulated and analyzed extensively.

CHAPTER 5
EVALUATION AND CHARACTERIZATION OF THE DUAL-BIOTIC SYSTEM

5.1 Introduction

Antibiotic therapy has been proven to be vital in the treatment and prevention of life-threatening conditions. As highlighted, this treatment however often results in unwanted side effects such as allergic reactions, nausea, vomiting as well as antibiotic-associated diarrhea, with probiotic supplementation being the first line prophylaxis for this condition (Holzapfel *et al*., 1998; D'Souza *et al*., 2002; Penner *et al*., 2005). The effectiveness of probiotic therapy is heavily reliant on patient compliance with patients required to ingest the probiotic product approximately two hours after the antibiotic formulation. The proposed Dual-Biotic System was therefore formulated to prevent probiotic treatment failure as a result of the simultaneous administration of probiotics with antibiotics. With the active ovalbumin granule system containing *L. acidophilus* statistically optimized in Chapter 4, this optimized system was advanced into a delayed release system that *in vivo* would allow for the absorption of the antibiotic prior to the release of the probiotic.

For the development of the delayed release system, glyceryl mono-stereate (GMS), a hydrophobic erodible organic molecule, was utilized to prevent physical contact between the antibiotic and probiotic bacteria (Lu *et al*., 2007; Laila *et al*., 2009). A hydrophobic polymer was required for the delayed release system as the optimized ovalbumin granules would swell considerably upon exposure to intestinal media (as seen in Chapter 3, Section 3.3.10). This swelling would therefore allow for the movement of pancreatic enzymes into the ovalbumin matrix with probiotic release thereafter taking place (as stated in Chapter 3, Section 3.3.14). This would ultimately result in an uncontrolled release of probiotic bacteria from the delayed release system leading to formulation failure. For this reason, the chosen polymer would have to be both erodible and hydrophobic in nature to cater to the requirements of the Dual-Biotic System. The low melting point of GMS (55-60°C) was also considered advantageous as the heat generated during compression would allow for the easier coating of the optimized granules through a modified melt granulation technique. Granules have been noted in literature to have low coating properties when compared to other dosage forms (Wen and Park, 2010). Once formulated and evaluated, the delayed release system was incorporated into a gastro-resistant hard gelatin capsule containing amoxicillin in the form of amoxicillin trihydrate. The use of a gastro-resistant capsule allowed for the site-specific delivery of the Dual-Biotic System to the small intestine leading to a more

effective, predictable and reproducible oral system that would not be dependent on the varying gastric emptying times associated with humans and animals.

As with all novel and innovative drug delivery systems, extensive *in vitro* and *ex vivo* characterization analyses were performed to ascertain the release, physicochemical and physicomechanical properties of the delivery system as well as to determine the adhesion and protective properties of *L. acidophilus* prior to the conduction of *in vivo* studies in Large White pigs. As previously described, probiotic adhesion to intestinal mucosal binding sites is vital for the ingested bacteria to provide functional health benefits to a patient. These analyses would therefore ensure the effectiveness of the formulated systems, as well as allow for the optimal usage of research resources such as animal models.

This chapter therefore provides the design and formulation of the delayed release system with advancement into the Dual-Biotic System. Extensive *in vitro* characterization of the optimized, delayed release and Dual-Biotic systems is performed in addition to accelerated stability studies. These analyses furthermore details the relationship between the individual systems with emphasis on the effects of formulation modifications on the physicomechanical, structural and thermal characteristics of the formulations as well as their effect on bacterial stability over time.

5.2 Materials and Methods

5.2.1 Materials

Lactobacillus acidophilus ATCC 314 was purchased from Davies Diagnostics (Randburg, Gauteng, South Africa). Raw egg ovalbumin obtained from commercial chicken eggs (Nulaid, Grade 1, Grain Fed, Johannesburg, Gauteng, South Africa). Genipin, amoxicillin trihydrate, penicillinase derived from *Bacillus cereus,* kollicoat MAE100P, pancreatin from porcine pancreas and pepsin from porcine gastric mucosa were purchased from Sigma-Aldrich (St Louis, MO, USA). BacTitre-Glo microbial cell viability assay was purchased from Promega (Madison, WI, USA). Glyceryl monostereate was purchased from Holpro Analytics (Johannesburg, Gauteng, SA). All other chemicals were of analytical grade and used as received.

5.2.2 Formulation of the delayed release system

Upon statistical optimization of the ovalbumin granule system, GMS was employed to delay the release of *L. acidophilus* by two hours to avoid contact with chemical entities such as antibiotics. To determine the ratio of GMS to optimized granules required for a 2 hour delayed release, granules composing of ratios ranging from 1:1 to 5:1 were formulated and analyzed using *in vitro* probiotic release analysis. The encapsulated granules were formulated through the dry granulation method by mixing the respective amount of GMS with the optimized granules and compressing the mixture at a force of 3 tons. The probiotic spiking time was thereafter determined through evaluation of the respective release profile with the ratio required for a 2 hour delayed release determined through linear regression. This was utilized due to the single variable employed (GMS).

5.2.3 Fourier Transform Infrared Spectroscopy

FTIR spectroscopy was undertaken on the optimized system, GMS and the delayed release system as outlined in Chapter 4, Section 4.2.4.1 to ascertain the structural effects of the addition of GMS on the optimized system. This was undertaken to ensure that the structural properties of the optimized system were maintained throughout the formulation of the delayed release system.

5.2.4 Thermodynamic measurements

DSC analysis was conducted on the non-crosslinked, optimized and delayed release systems to determine their thermal properties as well as their resistance to temperature changes. This analysis would also determine if the addition of GMS had affected the thermal properties of the optimized system. Thermodynamic measurements were undertaken on a Differential Scanning Calorimeter (DSC, METTLER Toledo DSC1, STAR^e System, Switzerland). In each case, the respective samples were sealed in aluminum DSC pans (40µL) and subjected to calorimetric analysis at a heating rate of 10℃ per minute over a temperature range of 25℃ to 300℃. Samples of 6.1mg and 6.5mg were utilized for the optimized and delayed release systems respectively.

5.2.5 Moisture content analysis

Moisture content of the optimized and delayed release systems was determined using a using a Karl-Fisher Volumetric Titrator as described in Chapter 3, 3.2.6. For this analysis, 0.0111g and 0.0377g of the optimized and delayed release systems respectively was added to titrator prior to the conduction of the experiment.

5.2.6 Surface morphology analysis

Surface morphology of the optimized and delayed release systems was analyzed utilizing a scanning electron microscope (FEI Phenom™, Hillsboro, Oregon, USA). Samples were mounted on aluminum stubs with carbon tape and gold sputter coated with argon gas under a vacuum of 0.5 Torr for 60 sec utilizing a SPI-MODULE™ Sputter Coater and SPI-MODULE™ Control (SPI Supplies, Division of Structure Probe Inc., West Chester, PA, USA). For the production of high resolution images, an electron acceleration charge of 20 kV was used with visualization occurring within the SEM unit.

5.2.7 Porositometric analysis

Surface porositometric analysis of the optimized and delayed release systems were undertaken utilizing an ASAP 2020 Porositometer (Micromeritics Instrument Company (Pty) Ltd., Norcross, GA, USA) equipped with research grade ASAP 2020 V3.01 software where quantifiable aspects of the individual system's porous nature such as the surface area, pore diameter, total pore volume and bulk and absolute densities were determined. Briefly, the samples were treated with a heat gradient and were evacuated with Nitrogen gas (N_2) (hard-sphere diameter = 3.860Å; molecular cross-section = 0.162nm^2) for the removal of surface moisture and gas particles prior to analysis. The optimized and delayed release systems samples were covered in an isothermal jacket and degassed for approximately 18 hours. The degassed samples were transferred to the analysis port and immersed in liquid nitrogen prior to the porosity analysis. The Barrett, Joyner and Halenda (BJH) and Brunauer-Emmett-Teller (BET) adsorption and desorption relationships were subsequently generated. The parameters employed for evacuation and heating phases of the porosity analysis are listed in Table 5.1.

Table 5.1: Parameters employed for the evacuation and heating phases of the porosity analysis.

Parameter	Rate/Target
Evacuation Phase	
Temperature ramp rate	10℃/min
Target temperature	40℃
Evacuation rate	50.0mmHg/s
Unrestricted evacuation from	30mmHg
Vacuum set point	500 µmHg
Evacuation time	60 min
Heating Phase	
Temperature ramp rate	10℃/min
Hold temperature	30℃
Hold time	1320min

5.2.8 Magnetic Resonance Imaging of the delayed release system

MRI of the delayed release system was conducted over a 24 hour period in SIF utilizing a digital MARAN-i System configured with a DRX2 HF Spectrometer console set to predetermined parameters as stated in Chapter 3, Section 3.2.8.

5.2.9 Determination of the Carr's Compressibility Index, Angle of Repose and Matrix hardness of the delayed release system

The CCI and AoR of the delayed release system was determined through the methods as outlined in Chapter 4 and calculated utilizing Equations 4.1 and 4.2 respectively. The BHN of the delayed release system was determined through calculation as outlined in Chapter 3 utilizing Equation 3.1.

5.2.10 Friability analysis

Friability analysis on the optimized and delayed release granules was conducted utilizing the method as outlined by Mehta *et al*, (2012) with modifications. For this analysis, the respective granules were placed in a Friabilator with glass beads (diameter: 4mm) over a

period of 10 minutes at a rotational speed of 25rpm. The friability of the optimized and delayed release granules was determined using Equation 3.3.

5.2.11 Determination of the antibiotic effectiveness of amoxicillin against *L. acidophilus*

An antibiotic effectiveness assay was performed to determine the bactericidal effects of amoxicillin on *L. acidophilus*. This analysis would also confirm that during *in vitro* probiotic release studies, the concentrations of amoxicillin trihydrate present in solution would be effective against *L. acidophilus*. For this analysis, varying concentrations of amoxicillin trihydrate (from 0.01 to 1.0mg/mL) were placed into wells of Thioglycolate agar containing *L. acidophilus* (100µL culture in 20mL molten agar). The antibiotic effectiveness was determined by incubation of the agar with antibiotic at 37℃ for 24 hours under anaerobic conditions and visually determining the diameter of the zones of inhibition after incubation. This analysis was conducted in triplicate for accuracy of results.

5.2.11.1 *Determination of the in vitro hydrolysis of amoxicillin through the addition of penicillinase*

Whilst absorption of amoxicillin would occur within a 2 hour period *in vivo*, this could not be accurately replicated *in vitro* without the removal of the delivery system and placing it in fresh *in vitro* dissolution media during the conduction of the release analysis. This would, however, result in residual amoxicillin solution being transferred to the fresh media which due to the high potency of amoxicillin to *L. acidophilus* would result in the destruction of bacterial cells. Penicillinase was therefore utilized in this study to inactivate amoxicillin in dissolution media prior to the release of *L. acidophilus*. Manufacturer specifications state that one unit of penicillinase will hydrolyze 1.0µmole of amoxicillin per minute at pH 7.0 at 25℃. To validate the effectiveness of penicillinase, a solution of 10units/mL was formulated and added to a solution of 0.28mg/mL of amoxicillin (250mg amoxicillin in 900mL SIF). Through calculation, it was determined that complete inactivation of amoxicillin would occur at approximately 70 minutes after the addition of penicillinase. This was confirmed by removing 100µL of inactivated solution and placing it within a well of Thioglycolate agar containing *L. acidophilus*. Penicillinase effectiveness was determined through incubation of the agar at 37℃ for 24 hours under anaerobic conditions and visually determining the diameter of the zones of inhibition after incubation. As controls, amoxicillin solution prior to the addition of penicillinase was placed into well plates and incubated as stated. This analysis was conducted in triplicate for accuracy of results.

5.2.12 Dissolution modeling of the delayed release system

Dissolution modeling was undertaken on the *in vitro* probiotic release data of the delayed release system utilizing the zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg and Korsmeyer-Peppas models. The zero order rate equation (Equation 5.1) best describes systems where the drug release rate is independent of its concentration. These systems are generally non-disintegrating and release drug slowly over a period of time (Costa and Lobo, 2001). The first order equation (Equation 5.2) describes a concentration-dependent release rate from a system and can be further utilized for the prediction of absorption and elimination of certain drugs (Costa and Lobo, 2001; Bourne, 2002; Dash *et al*., 2010). The Higuchi model is utilized to describe the release of drugs from an insoluble matrix or low soluble drugs in solution where the drug in the matrix is considered higher than the drug in solution (Equation 5.3). It is represented as a square root of time dependent process based on Fickian diffusion (Higuchi, 1963). The Hixson-Crowell cube root law (Equation 5.4) best describes release from systems which is dependent on changes in surface area and diameter of the delivery system (Hixson and Crowell, 1931). The Hopfenberg model (Equation 5.5) is utilized to predict drug release from a surface eroding system and is dependent on the surface area remaining constant during the degradation process (Dash *et al*., 2010). The Korsmeyer-Peppas model (Equation 5.6) is a simple mathematical relationship between exponential release and time elapsed and can be utilized in various modified release delivery systems.

$$C = k_0 t \qquad \text{Equation 5.1}$$

Where:
Where k_0 is the zero-order rate constant and
t is the time elapsed.

$$\log C_0 = \log C_0 - \frac{k_t}{2.303} \qquad \text{Equation 5.2}$$

Where:
C_0 is the initial concentration of drug and
k_t is the first order constant.

$$Q = k_H t^{1/2} \qquad \text{Equation 5.3}$$

Where:

Where Q is the drug dissolved,

k_H is the constant reflecting the design variables of the system and

t is the time elapsed.

$$Q_0^{1/3} - Q_t^{1/3} = k_{HC} t \qquad \text{Equation 5.4}$$

Where:

Q_0 is the initial amount of the drug in the delivery system,

Q_t is the amount of drug released in time t and

K_{HC} is the rate constant.

$$\frac{\mathrm{Mt}}{\mathrm{M}\infty} = 1 - [1 - \frac{\mathrm{k_{HB}t}}{\mathrm{C_L a}}]^{\mathrm{n}} \qquad \text{Equation 5.5}$$

Where:

M_t/M_∞ is the fraction of drug released,

K_{HB} is the zero order rate constant,

C_L is the initial drug in the system,

a is half the system thickness and

n = 1 (slab), 2 (cylinder) or 3 (sphere).

$$\frac{\mathrm{Mt}}{\mathrm{M}\infty} = k_{KP} t^n \qquad \text{Equation 5.6}$$

Where:

M_t/M_∞ is the fraction of drug released,

K_{KP} is the rate constant,

t is the time elapsed and

n is the release exponent.

5.2.13 X-Ray diffraction analysis

X-Ray diffraction (XRD) was undertaken on the optimized and delayed release systems as well as a mixture of GMS and amoxicillin trihydrate utilizing a X-Ray Diffractometer (Rigaku Miniflex 600, Rigaku Corporation, Matsubara-cho, Akishima-shi, Tokyo, Japan) containing a 10mm Incident Height Slit (IHS), 1.25° Divergence Slit (DS), 13mm Solar Slit (SS) and 13mm Receiving Slit (RS). This was performed to ascertain the effect of GMS on the crystalline structure of ovalbumin and amoxicillin trihydrate. All experimental procedures were conducted at room temperature with a start angle of 3°, stop angle of 140°, step of 0.1° and speed of 30° per minute. The theta/2-theta values were determined through use of integrated X-ray powder diffraction software (PDXL 2.1, Rigaku Corporation, Matsubara-cho, Akishima-shi, Tokyo, Japan) with any changes in crystallinity thus determined.

5.2.14 Construction of a calibration curve for amoxicillin trihydrate

A calibration for amoxicillin trihydrate was constructed utilizing serial dilutions of known concentrations ranging from 0.1 to 0.5mg/mL dissolved in SIF. Samples were analyzed utilizing a Nanophotometer at a wavelength of 273nm with the absorbance of the respective samples determined. A calibration curve of absorbance readings versus amoxicillin trihydrate concentration was constructed as per Beer-Lamberts Law.

5.2.15 Formulation and *in vitro* probiotic release analysis of the Dual-Biotic System

The Dual-Biotic System was formulated using the delayed release system (optimized ovalbumin granules encapsulated with a ratio of 1:94 ovalbumin to GMS) placed in a hard gelatin capsule containing amoxicillin trihydrate granules (formulated through dry granulation at 0.5 tons) equivalent to 250mg amoxicillin base. *In vitro* probiotic release analysis was conducted using the method described in Chapter 3, Section 3.2.6.7 with modifications. To inactivate the amoxicillin within 2 hours, penicillinase was added to the dissolution media 30 minutes after conduction of the analysis. Release samples were analyzed using UV spectroscopy for amoxicillin and luminescence for *L. acidophilus*.

For the formulation of the site-specific delivery system, the Dual-Biotic System was coated with a gastro-resistant polymer coating solution (Kollicoat MAE100P). Kollicoat MAE100P was formulated to the requirements of the delivery system by hydrating a 50% (w/v) MAE100P solution in distilled water for 4 hours and thereafter adding propylene glycol (10%v/w) as a plasticizer. Tartrazine Green dye was added to the coating solution to ensure complete coating of the gelatin capsule. To simulate transition through the gastrointestinal

tract, the site-specific Dual-Biotic System was placed in simulated gastric conditions at pH 1.2 for 2 hours and thereafter placed in simulated intestinal conditions at pH 6.8 for a further 4 hours. Release samples were analyzed using UV spectroscopy for amoxicillin and luminescence for *L. acidophilus*.

5.2.16 Accelerated stability analysis

Stability of the optimized, delayed release and Dual-Biotic systems were conducted utilizing the method as outlined by Silva *et al*. (2013) with modifications to ascertain the survival of *L. acidophilus* over storage conditions. For this analysis, the optimized, delayed release and Dual-Biotic systems were incorporated into hard gelatin capsules and placed in the sealed chamber of a humidifier (Labcon humidity chamber, Labmark, Johannesburg, South Africa) at 23ºC and 33% relative humidity for a period of 3 months. Samples were taken at monthly intervals and evaluated through luminescence as well as inoculation onto Thioglycolate agar. As controls, the optimized and delayed release systems were place into sterile petri dishes and placed in the humidifier to ascertain the effect of the hard gelatin capsule on cell viability. Further moisture content analyses were conducted on all samples utilizing the Karl-Fisher titrator as previously stated (Chapter 3, Section 3.2.5) to determine the effect of moisture uptake on cell viability.

5.3 Results and Discussion

5.3.1 Investigation of the ratio of GMS required for the delayed release system

GMS was added to the optimized granule system at various ratios ranging from 1:1 to 5:1 (GMS to Ova). The resultant formulations were subject to *in vitro* probiotic release analysis and the time taken for an initial spike in bacteria ATP determined through calculation. The initial spike would therefore determine the delayed release time of each ratio formulated. Results of this analysis through linear regression determined that an ideal concentration of 1.94:1 would allow for a delayed release of two hours. The ratio of 1.94:1 was therefore used for the formulation of the delayed release system incorporated into the Dual-Biotic System. The results of the delayed release times are listed in Table 5.2.

Table 5.2: Delayed release times of the various ratios of GMS formulations analyzed (n=3; SD≤0.38 in all cases).

Ratio (GMS to Ova)	Release Delay (hours)
1:1	1.300
2:1	2.050
5:1	4.800
1.94:1	2.000

5.3.2 Structural evaluation of the optimized and delayed release systems

FTIR spectra of the optimized system, GMS and the delayed release system are depicted in Figure 5.1. GMS is a mixture of glyceryl ester of fatty acids made up of approximately 30% glyceryl monopalmitate and 5% glyceryl monomyristate and is composed of a hydrophilic and a lipophilic portion (Ranken *et al*., 1997; Kamble *et al*., 2010). Evaluation of the GMS FTIR spectra (Figures 5.1b) revealed peaks at 3305cm^{-1} and 2914cm^{-1} depictive of O-H and C-H stretching of the hydrophilic and lipophilic components of GMS respectively (Ranken *et al*., 1997; Ahire *et al*., 2012). A large transmittance peak was noted at 1729cm^{-1} due to C=O stretching of the hydrophilic portion. Other significant beaks were determined at the 1470 cm^{-1} and 1177cm^{-1} bands indicative of C-C vibrations primarily due to the lipophilic portion of the GMS molecule. Evaluation of the delayed release system detailed no significant changes in band widths or the formation of additional peaks determining that GMS did not have any structural effects on the optimized ovalbumin matrix. A comprehensive evaluation of the optimized granule system can be found in Chapter 4, Section 4.3.2.

Similarly, it was also necessary to determine the effect of GMS on the structural integrity of amoxicillin trihydrate. This was vital as a change in the chemical structure of a dosed antibiotic would diminish its therapeutic effectiveness, compromising the treatment regimen of the patient. FTIR spectra of amoxicillin trihydrate and the dual-biotic combination (GMS and amoxicillin trihydrate) are shown in Figure 5.2. Evaluation of the FTIR spectra of amoxicillin trihydrate detailed a transmittance peak at 3445cm^{-1} due to O-H and N-H stretching (Songsurang *et al*., 2011). Other peaks noted were at 1771cm^{-1} (depictive of C=O stretching of β- lactam ring), 1684cm^{-1} (as a result of C=O stretching of the amide), 1392cm^{-1} (C-H_3 vibrational bending) 1246cm^{-1} (C-N stretching, 1076cm^{-1} (C-O stretching) and 837cm^{-1} (bending vibration due to N-H) (Ghosh *et al*., 2009; Songsurang *et al*., 2011). The FTIR spectra of the dual-biotic combination revealed no significant peak formations or shifts detailing no chemical interactions or structural changes in the amoxicillin trihydrate matrix as

a result of the interaction with GMS. This therefore advocated the use of GMS in the Dual-Biotic System.

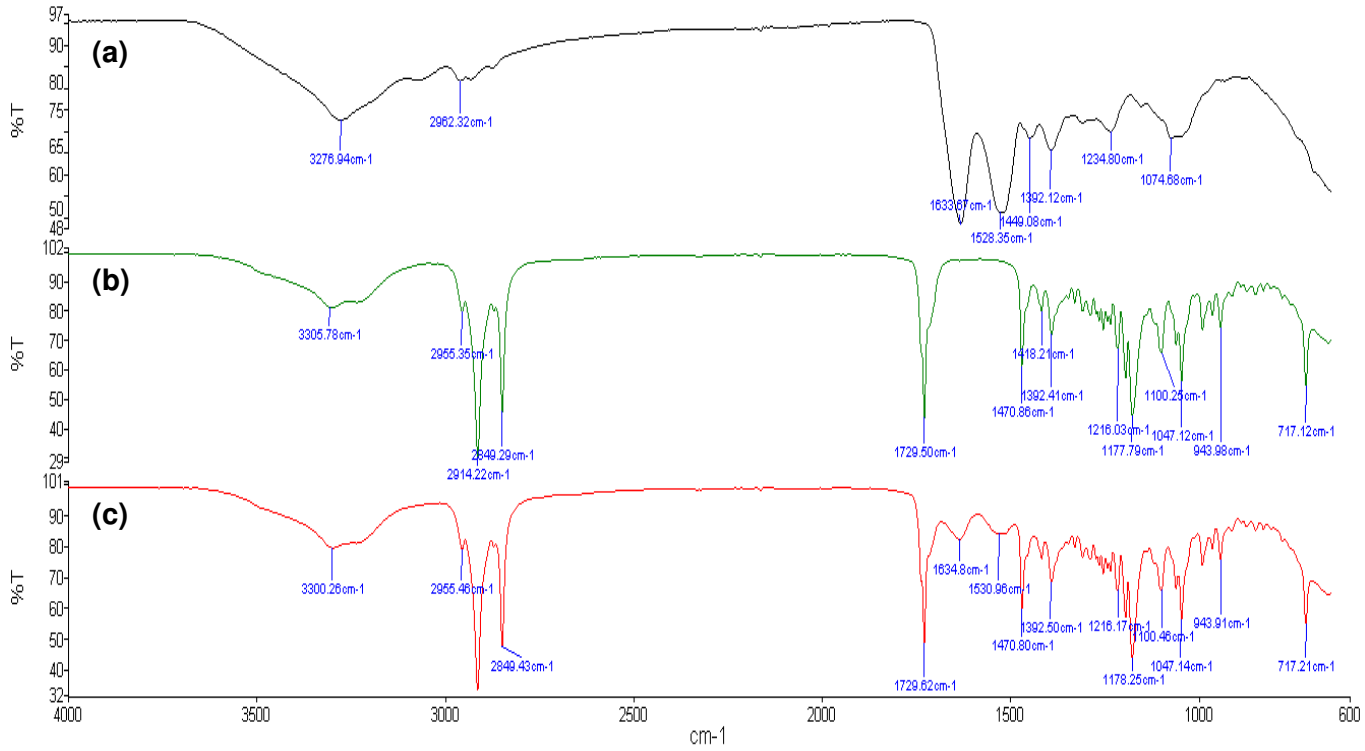


Figure 5.1: FTIR spectra of (a) the optimized system, (b) GMS and (c) the delayed release system.

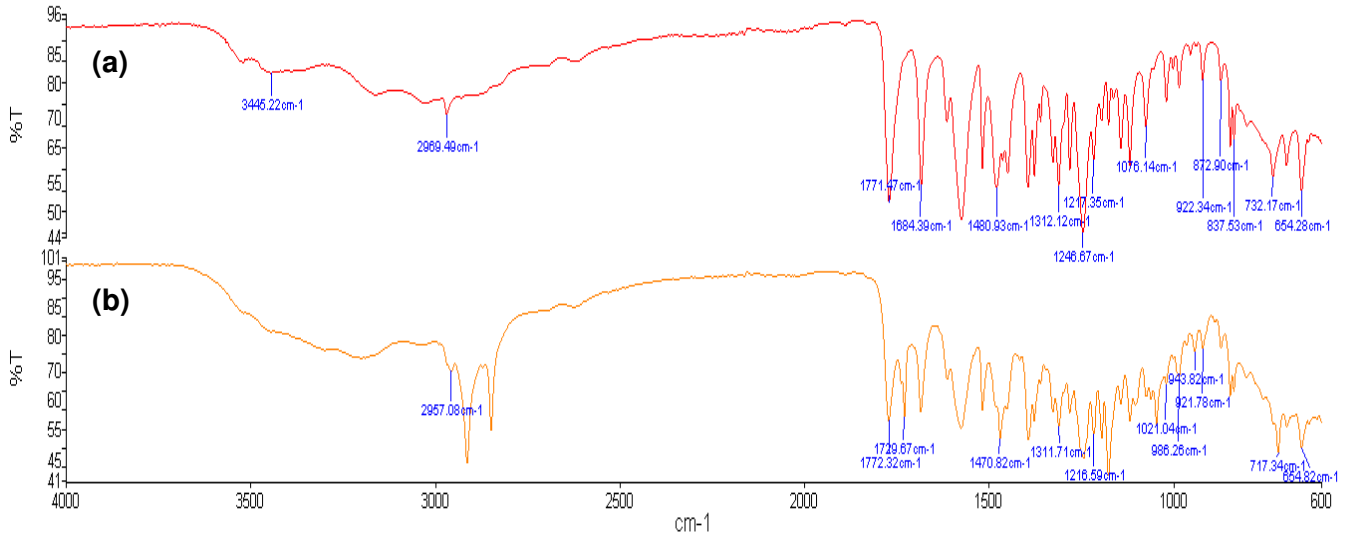


Figure 5.2: FTIR spectra of (a) amoxicillin trihydrate and (b) the Dual-Biotic System combination.

5.3.3 Thermal analysis of the non-crosslinked, optimized and delayed release systems

Scanning differential calorimetry (DSC) analysis determined the temperatures of thermal protein denaturation and degradation of the non-crosslinked, optimized and delayed release systems. Analysis of the non-crosslinked ovalbumin matrix determined an endothermic peak at 82.27℃ indicative of albumin denaturation without degradation, an endothermic peak of 218.64℃ representative of the initial phase of protein degradation and an exothermic peak at 290.05℃ noting protein decomposition (Figure 5.3a) (Jones et al., 2013). Comparison to the crosslinked ovalbumin system (Figure 5.3b) determined a shift in the endothermic denaturation peak to 106.40℃ with an increased initial endothermic degradation and exothermic decomposition peak at 221.77℃ and 294.96℃ respectively. The shift in the endothermic and exothermic peaks observed can be attributed to the crosslinking effects of genipin resulting in a more thermally stable structure being formed. DSC profiling of the delayed release system (Figure 5.3c) determined a lower endothermic peak at 85.40℃, which can be attributed to the melting point of GMS, an initial endothermic degradation peak at 217.52℃ and an exothermic decomposition peak of 266.91℃.

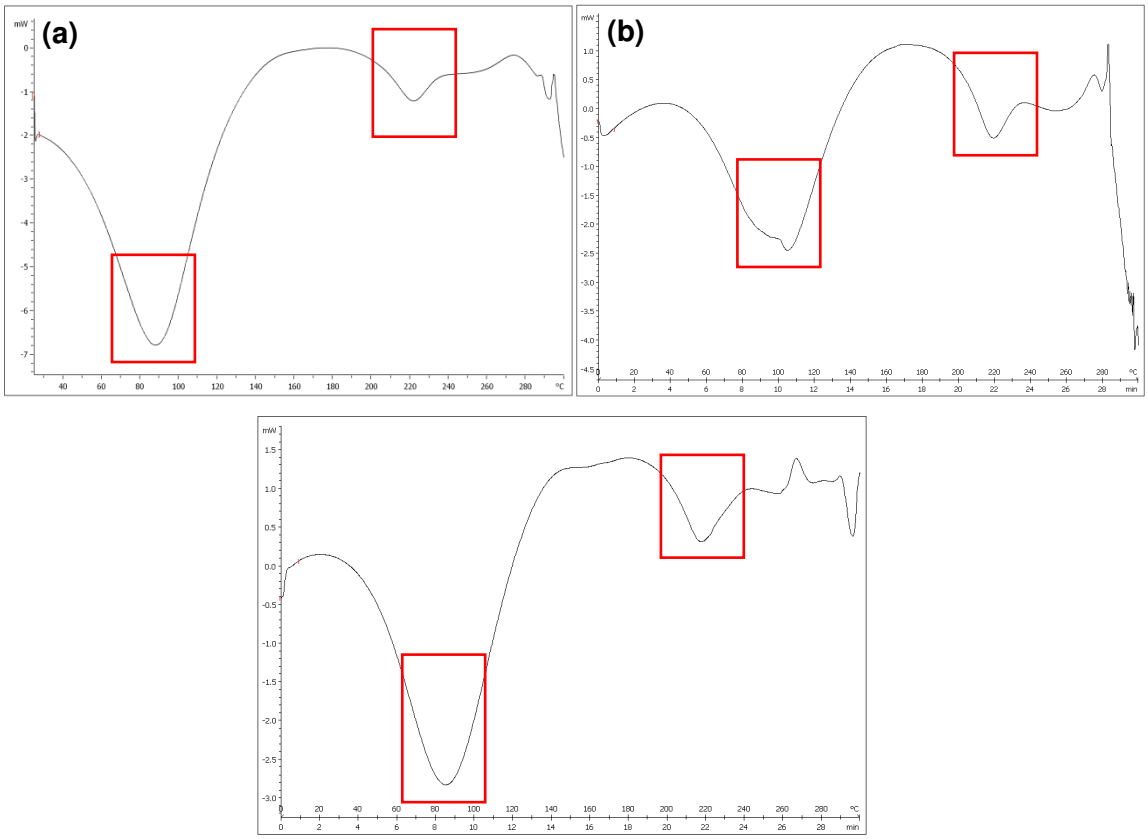


Figure 5.3: DSC thermograms of the (a) non-crosslinked, (b) optimized and (c) delayed release systems.

5.3.4 Moisture content evaluation

A low moisture content is ideal in a formulated granule system as the effect of excess moisture can be detrimental to the stability of the granules over time. This stability is of greater significance in probiotic bacteria as the addition of moisture significantly decreases the stability and viability of encapsulated bacteria over time (Vesterlund *et al*., 2012). Results of the moisture content analysis of the optimized system revealed a moisture content of 2.981% (Table 5.3). This moisture content was ideal (2.8-5.6%) for the preservation of bacterial viability and furthermore showed the effectiveness of the freeze-drying process in the formulation of the ovalbumin granules (Zayed and Roos, 2004). The moisture content of the delayed release system was, however, notably lower (1.392%). Whilst this was out of the ideal range for probiotic stability, the moisture content seen is acceptable due to the hydrophobic nature of GMS. The moisture content of GMS itself is significantly lower compared to the optimized system and due to the large ratio of GMS to ovalbumin, the moisture content diminished to outside the ideal levels. This analysis also confirmed that GMS would provide greater protection against moisture upon storage and whilst serving primarily as the delayed release mechanism, would improve storage stability as well.

Table 5.3: Moisture content of the optimized and delayed release systems.

Formulation	Moisture Content (%)
Optimized	2.981
Delayed Release	1.392

Moisture content analysis through Karl-Fisher titration also provides data on the residual and molecular moisture of the sample analyzed. Figure 5.4, the moisture content profile of the optimized system, illustrates that property. The residual moisture content is emitted first resulting in the large initial signal seen. As the titration proceeds, the signal becomes lower in which the molecular moisture, which is significantly less, is emitted. This analysis revealed two properties of the optimized system: that the residual moisture was the significant proportion of the total moisture emitted, but more importantly, that the molecular water, crucial for the survival of bacteria cells, was maintained.

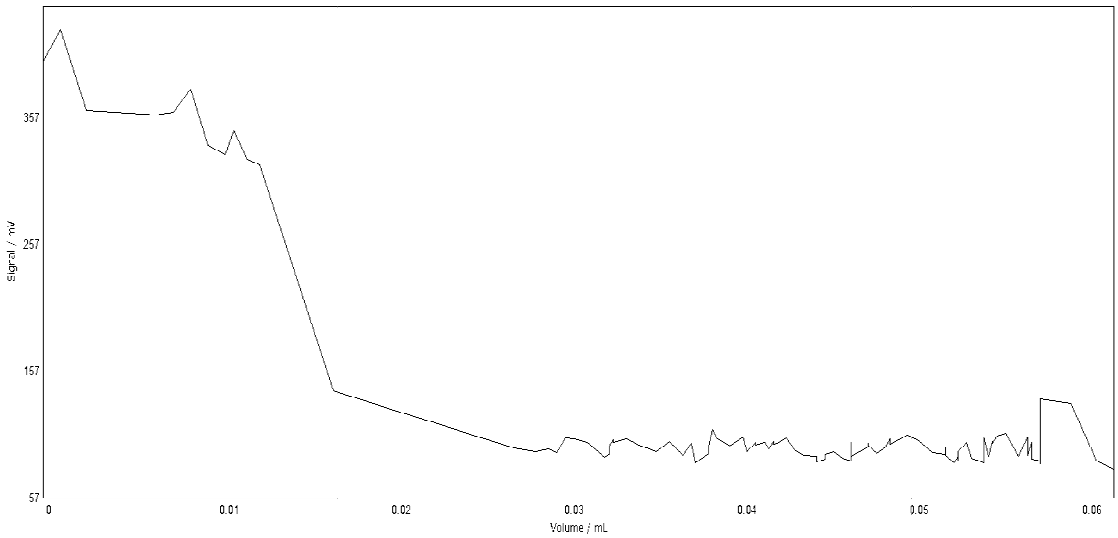


Figure 5.4: Karl-Fisher Titration profile of the optimized system.

5.3.5 Surface morphology evaluation

Surface morphology description using SEM analysis was employed on the optimized and delayed release systems. This analysis would determine not only the size of the granules formed but also the surface characteristics of the granules. Surface characteristics with regards to total GMS coverage as well pore size can be evaluated through these images. Results of this analysis showed an increase in the size of the delayed release system, which is due to the increase in granule composition by the addition of GMS as well as the granulation process being kept constant. Pore size can also be seen to decrease in the delayed release system which can be attributed to the melt granulation ability of GMS. The SEM images of the optimized and delayed release systems are shown in Figure 5.5a and 5.5b respectively.

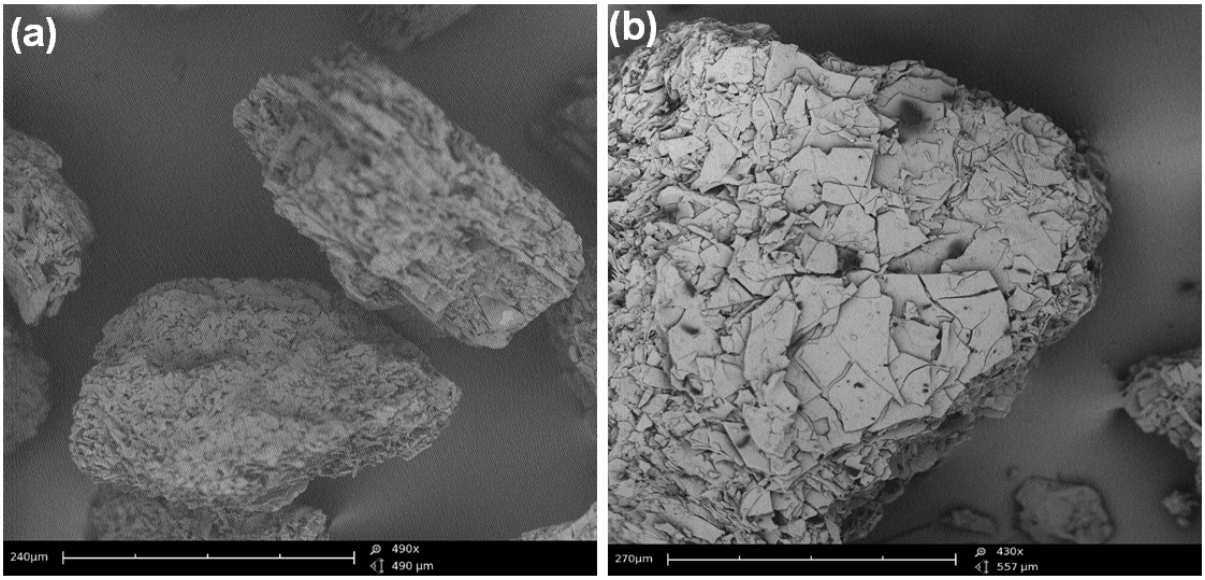


Figure 5.5: SEM images of (a) the optimized and (b) delayed release granule systems.

5.3.6 Surface porositometric analysis

The average pore size, the pore size distribution, the pore volume and the pore interconnections provided important data concerning the surface of the optimized and delayed release systems. Both samples demonstrated a type III isotherm with a type H4 hysteresis loop. There are various types of isotherms and hysteresis loops that are produced dependent on the affinity and amount of gas adsorbed onto a particular material in relation to its relative pressure. A type III isotherm is indicative of a multilayered material with a low adsorption at low pressure representing a material with a low gas affinity. The single point surface area of the optimized system was calculated to be 7.3504m²/g whilst the single point surface area of the delayed release system was determined to be 3.1906m²/g. This can be attributed to the optimized system being significantly smaller in size when compared to the delayed release system, as seen in Section 5.3.5, resulting in a greater surface area. The adsorption average pore width of the optimized system was calculated to be 29.4431Å with the average adsorption and desorption pore diameters being 49.809Å and 49.369Å respectively. The average adsorption pore width of the delayed release system was calculated to be -0.9445Å with the average adsorption and desorption pore diameters being 57.125Å and 50.835Å respectively. With regards to the pore volume, the optimized and delayed release systems were determined to have a single point adsorption of 0.016081cm^3/g and 0.006877cm^3/g, respectively. The large decrease in single point adsorption was due to the delayed release system being significantly less porous in nature when compared to the optimized system. This is significant as a decrease in surface porosity results in a decrease in water uptake preventing the hydration of the optimized system. This

in effect prevents the premature release of probiotic bacteria leading to a more predictable release profile.

Through the porosity analysis, it was determined that the delayed release system was significantly less porous in nature as compared to the optimized system. The decrease in porosity also indicates that erosion takes place on the delayed release system surface as opposed to core hydration and dissolution further proving the erosion capability of GMS. The BET Surface Area and Isotherm Log plots are shown in Figures 5.6 and 5.7 for the optimized system and Figures 5.8 and 5.9 for the delayed release system, respectively.

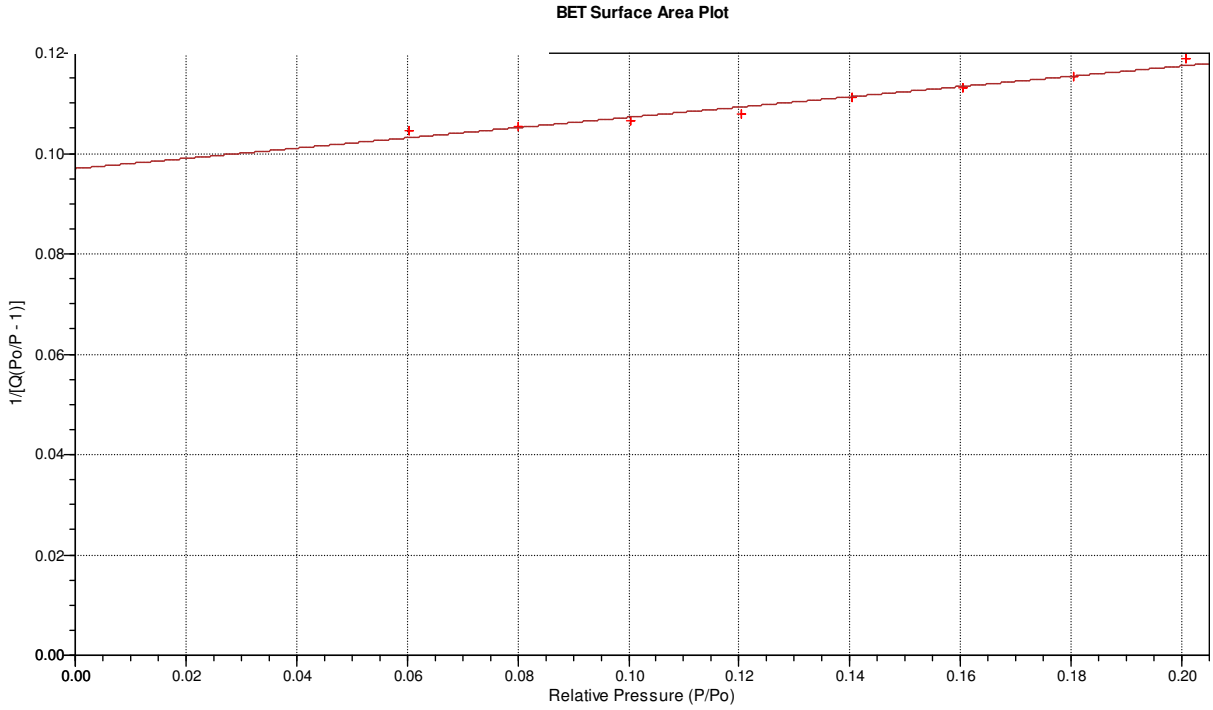


Figure 5.6: BET surface analysis plot of the optimized granule system.

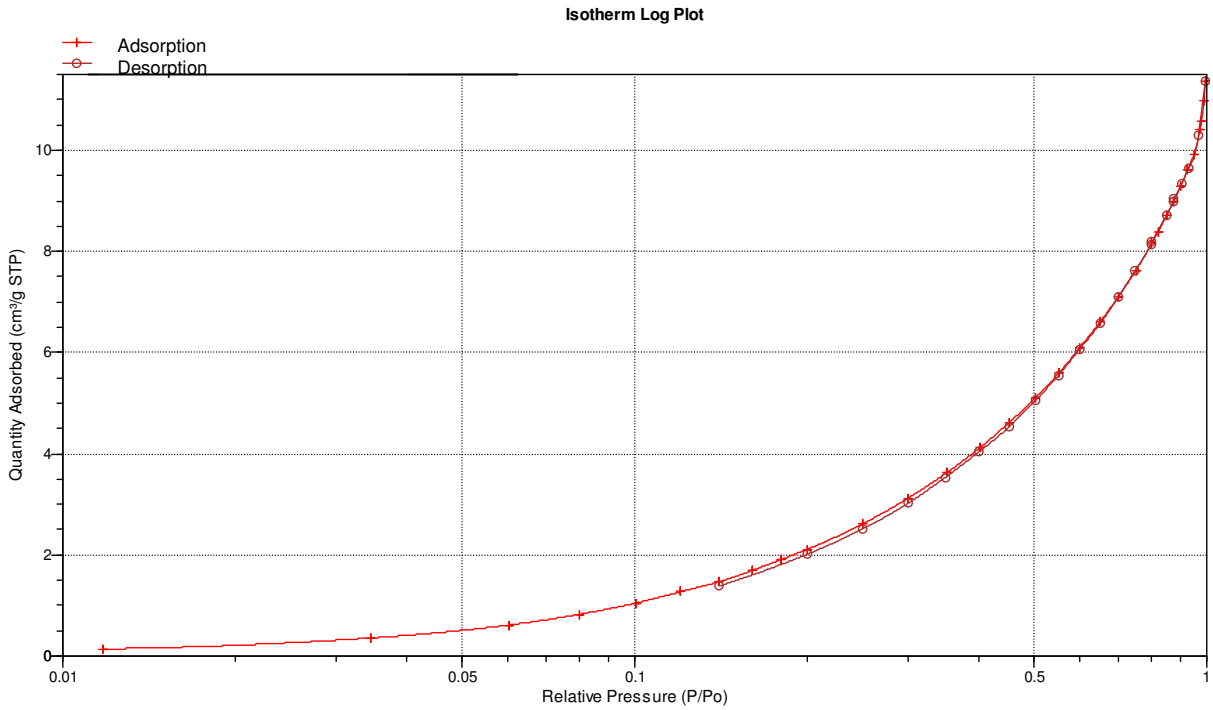


Figure 5.7: Isotherm log plot of the optimized granule system.

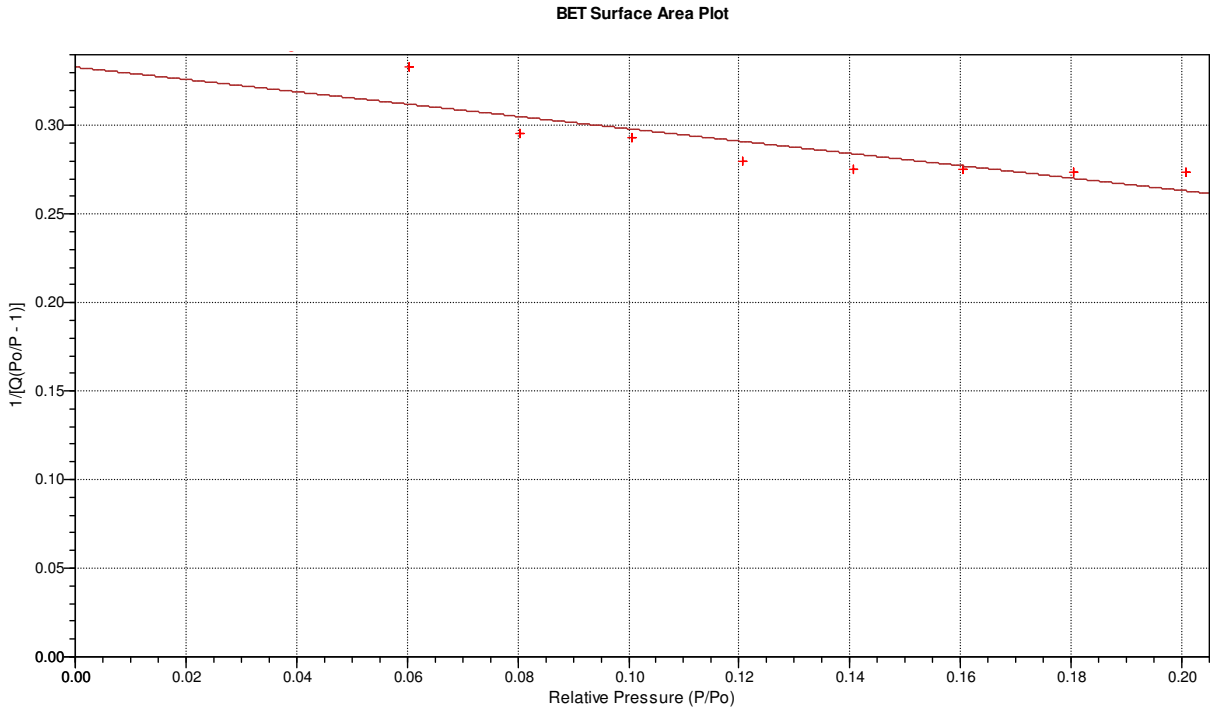


Figure 5.8: BET surface analysis plot of the delayed release system.

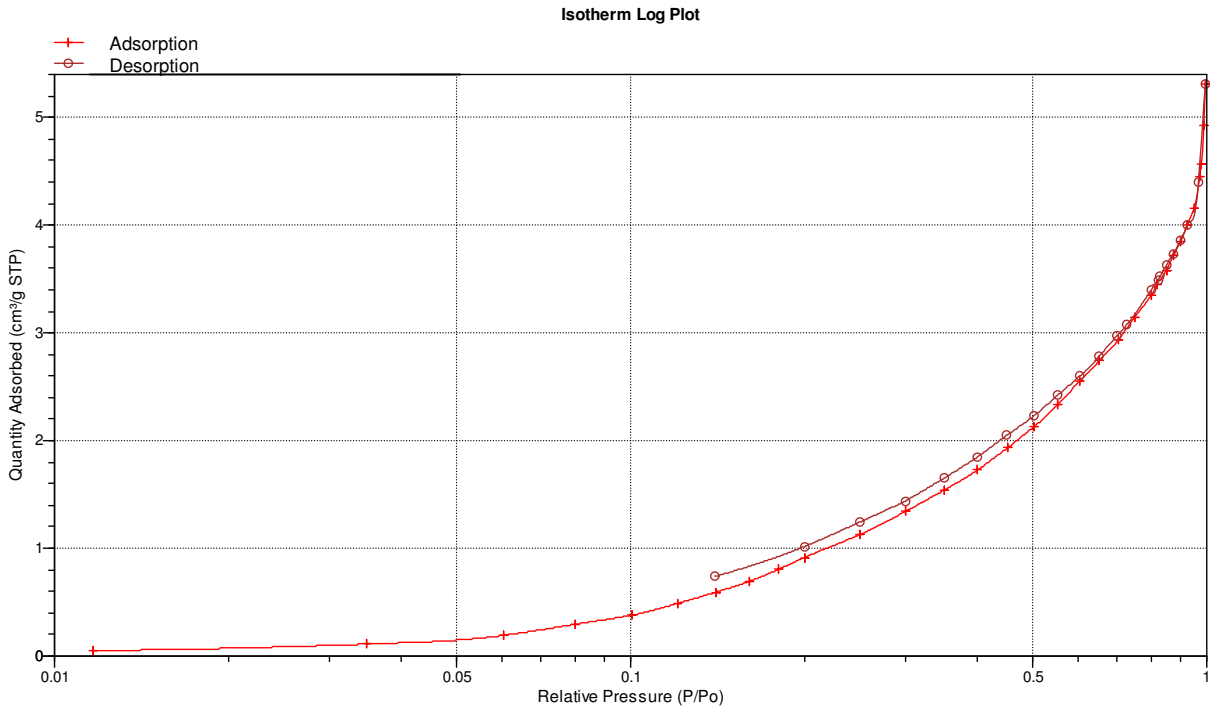


Figure 5.9: Isotherm log plot of the delayed release system.

5.3.7 Magnetic resonance imaging

Magnetic resonance imaging of the delayed release system revealed the constant hydration of the system over a period of 2 to 3 hours (Figure 5.10a to 5.10e) with minimal swelling noted. The dark portions of the system (primarily in Figure 5.10a to 5.10c) depict the compressed anhydrous matrix, with the grey to white portions (in Figure 5.10d to 5.10f) depicting complete hydration. The hydrophobic nature as well as the aggregating ability of GMS can also be visualized with the delayed release system maintaining its original form even after matrix hydration has taken place (Figure 5.10e). This is of significance as it visually confirms that upon the addition of dissolution media, the delayed release system mildly aggregates accordingly due to the hydrophobic nature of GMS. The result of particulate aggregation is a decreased gastrointestinal transit time, when compared to free particulates which moves through the GIT unrestricted (Krishna and Yu, 2008). This will be beneficial in the Dual-Biotic System to allow for probiotic release in the upper portion of the small intestine.

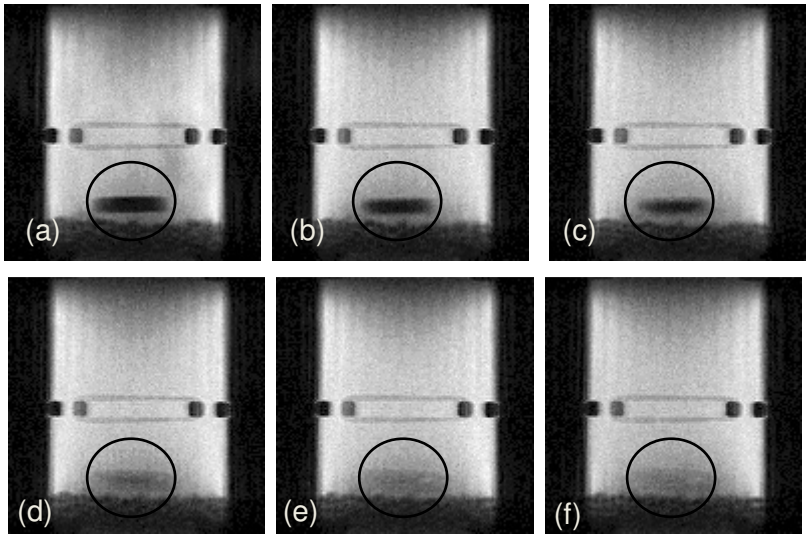


Figure 5.10: Magnetic Resonance Images of the delayed release system at (a) 0, (b) 30, (c) 60, (d) 120, (e) 180 minutes and (f) 240 minutes.

5.3.8 Evaluation of the Carr's Compressibility Index, Angle of Repose and Matrix hardness of the delayed release system

The CCI and AoR of the delayed release system were determined to be 6.94 and 5.71 respectively. The calculated values of CCI an AoR were both in the excellent range of flow properties as stated in Chapter 4, Section 4.3.3 confirming that good to excellent granule flow properties were maintained throughout. It can therefore be stated that through the excellent flow properties of the delayed release system and when taken in conjunction with the other CCI and AoR values determined in Chapter 4, Sections 4.3.3.3 and 4.3.3.4 respectively, that the ovalbumin derived granules can be utilized in large scale manufacture to produce capsule systems of an effective, predictable and reproducible nature. The BHN value of the delayed release system was also determined to be 2.44, which was higher than that determined for the optimized system but, however, still within the range determined in the Box-Behnken experimental design to be effective for *L. acidophilus* delivery. The higher BHN value can, however, be attributed to the increased in compression force utilized in the formulation of the delayed release system.

5.3.9 Friability of the optimized and delayed release systems

Friability evaluation of the optimized and delayed release granule systems revealed a friability of 0.53 and 0.65% respectively. Both of these values are within the required friability limit of 1% as outlined by the USP, highlighting the robust nature of the granules formulated and their ability to resist external forces during storage.

5.3.10 The bactericidal effects of amoxicillin trihydrate

The antibiotic efficacy assay performed on amoxicillin trihydrate revealed the potency of the antibiotic against *L. acidophilus*. Results of this analysis revealed that at concentrations ranging from 0.01 to 1mg/mL, amoxicillin trihydrate successfully inhibited its growth after the designated incubation period (Table 5.4). This result was in correlation with previous studies analyzing the antibiotic effectiveness of amoxicillin against probiotic bacteria where the MIC value was determined to be ≤2µg/mL (Felten *et al*. 1999; Chou *et al*., 2004). This therefore verified that during probiotic release analysis the amoxicillin concentration (0.28mg/mL) would have bactericidal effects on *L. acidophilus* cells and through the protection of contact between *L. acidophilus* and amoxicillin trihydrate, the effectiveness of the Dual-Biotic System could be determined.

Table 5.4: The bactericidal effects of amoxicillin trihydrate against *L. acidophilus*.

Concentration (mg/mL)	Bactericidal Effect
1.0	Positive
0.1	Positive
0.01	Positive

5.3.10.1 *Amoxicillin hydrolysis by penicillinase*

Hydrolysis of amoxicillin by penicillinase was crucial to inactivate the antibiotic prior to the release of the probiotic. As per manufacturer's specification, the penicillinase should inactivate amoxicillin in solution if added 30 minutes after conduction of the analysis. This time period would ultimately prove the effectiveness of the delivery system to protect the probiotic from the antibiotic and would also determine if formulation failure had occurred. Results of this analysis revealed that amoxicillin was successfully deactivated by penicillinase within the required 90-minute period and could be utilized as an inactivation tool for drug and probiotic release analysis of the Dual-Biotic System. The control of the amoxicillin solution without penicillinase revealed the potent bactericidal effects of amoxicillin against *L. acidophilus*.

5.3.11 *In vitro* probiotic release of the delayed release system

In vitro probiotic release analysis was conducted on the delayed release system to determine the effectiveness of the GMS encapsulation for the protection of the optimized

system against the bactericidal effects of amoxicillin. Penicillinase was added 30 minutes after conduction of the analysis to inactivate the amoxicillin to simulate the absorption of amoxicillin (Berney and Francioli, 1990). Results of this analysis determined that the delayed release system successfully delayed the release of *L. acidophilus* by two hours, when delivered concurrently with amoxicillin, with MPC reached within a 6 hour period (Figure 5.11). This result confirmed the release profile of the optimized system after encapsulation within the delayed release system. A further release analysis on the delayed release system without the addition of amoxicillin showed a greater bacterial cell count after the test period; however, this can be attributed to lack of total encapsulation by the GMS due to the granulation process employed. This resulted in the exposure of probiotic bacteria to the amoxicillin prior to the intended two hour period, resulting in the decreased cell count noted. The control of the *in vitro* probiotic release analysis on the delayed release system without the use of penicillinase showed a bacterial cell count of less than 0.1×10^8 cells attributed to the death of the probiotic bacteria in solution by amoxicillin. This control therefore confirms the bactericidal effects of amoxicillin on *L. acidophilus*, as determined in Section 5.3.10, and validates the use of penicillinase in solution. This release analysis therefore confirmed the protective properties of the delayed release system and validated its use in the final gastro-resistant Dual-Biotic System.

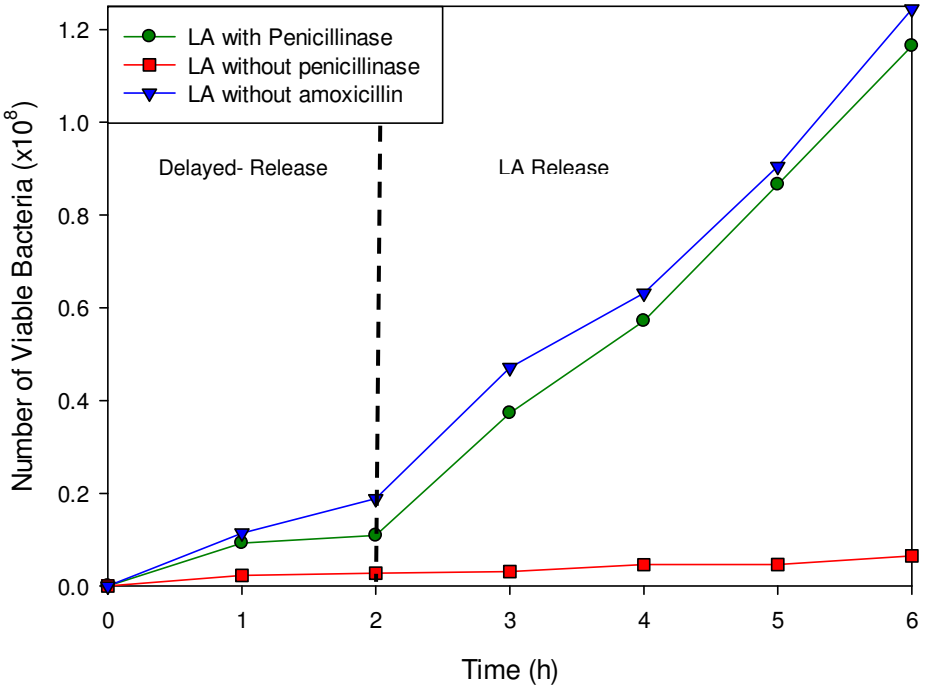


Figure 5.11: *L. acidophilus* (LA) probiotic release profile of the delayed release system (n=3; SD≤ 0.017 in all cases).

5.3.12 Dissolution modeling of probiotic release from the delayed release system

Kinetic modeling of the delayed release system probiotic release profile was undertaken in order to ascertain the best model of fit. The significance of this analysis is to provide a greater understanding of the release mechanism from the delayed release system that can serve as a prerequisite to *in vivo* studies. The zero order, first order, Higuchi, Hixson-Crowell, Hopfenberg and Korsmeyer-Peppas models were therefore employed for this purpose. The results from the kinetic analysis are represented in terms of Akaike information criteria (AIC) and Model selection criteria (MSC) calculated as per Equations 5.7 and 5.8 respectively. AIC attempts to determine the model that explains release data with a minimum of free parameters with the lowest AIC value being most ideal. The MSC is an adapted reciprocal value of the AIC with the highest value being most significant in model selection.

$$AIC = -2L_R + 2_S \qquad \text{Equation 5.7}$$

Where:

L_R is the restricted log-likelihood function evaluated at final fixed parameter estimates and the final variance parameter estimates and

s is the rank of the fixed effects design matrix X plus the number of parameters in θ

$$\ln\left(\frac{\Sigma_{t=1}^{n} w_i\,(y_{i\_obs} - y_{\_obs})^2}{\Sigma_{t=1}^{n} w_i\,(y_{i\_obs} - y_{i\_pre})^2}\right) - \frac{2p}{n} \qquad \text{Equation 5.8}$$

Where:

W_i is the weighting factor,

$yi_{\_obs}$ is the observed y value,

$yi_{\_pre}$ is the predicted y value,

$y_{\_obs}$ is the mean of all observed y-data points,

p is the number of parameters in model and

n is the number of data points.

Results of the kinetic analysis revealed that probiotic release from the delayed release system was best described by the Korsmeyer-Peppas model. This model had the greatest linearity (R^2 = 0.995), the lowest AIC and the highest MSC value when compared to the other models employed (Table 5.5). The composite probiotic release curves of the models employed are shown in Figure 5.12, where the Korsmeyer-Peppas model was determined to

have the greatest predictability when comparing predicted fractional probiotic release with observed values.

Table 5.5: Release data generated from the dissolution analysis models.

Dissolution Model	Constant	AIC*	MSC#	R^2 value
Zero Order	K_0 = 18.042	48.201	2.298	0.940
First Order	K_1 = 0.291	57.886	0.915	0.762
Higuchi	K_H = 35.856	58.837	0.779	0.727
Hixson-Crowell	K_{HC} = 0.083	56.232	1.151	0.812
Hopfenberg	K_{HB} = 0.196	49.703	2.084	0.944
	n value = 0.625			
Korsmeyer-Peppas	K_{KP} = 7.740	33.329	4.423	0.995
	n value = 1.542			

* AIC = Akaike information criteria
# MSC = Model selection criteria

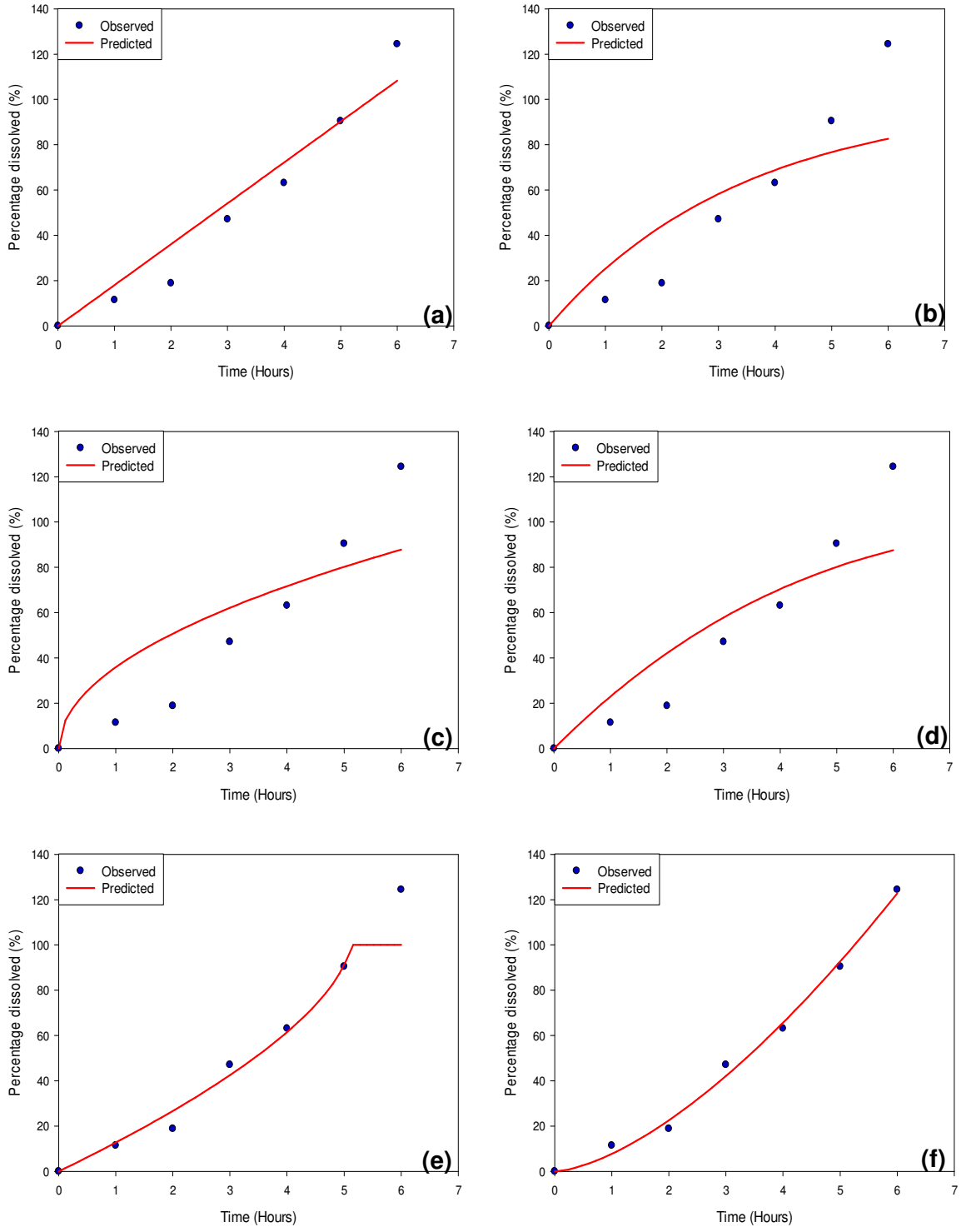


Figure 5.12: Observed versus predicted probiotic release profiles generated by the (a) Zero Order; (b) First Order; (c) Higuchi; (d) Hixson-Crowell; (e) Hopfenberg and (f) Korsmeyer-Peppas models.

5.3.13 X-ray diffraction analysis

Evaluation of the crystalline properties on the addition of genipin to ovalbumin revealed a similar θ value (36.595°with a higher intensity (554661 cps) when compared to the θ value of

non-crosslinked ovalbumin (36.782° and 319099 cps). The θ values were calculated through Bragg's law as stated by Equation 5.9. This confirmed that on the addition of genipin, a greater crystalline structure was formed due to the formation of crosslinks with the structure of ovalbumin largely maintained in this process.

$$n\lambda = 2d \sin\theta \qquad \text{Equation 5.9}$$

Where:
n is an integer,
λ is the wavelength of the incident wave,
d is the spacing between the planes in the atomic lattice and
θ is the incident angle between the incident ray and the scattering planes.

Determination of the crystalline properties of amoxicillin prior to and after the interaction with GMS revealed similar θ values (36.662°, 64966 cps and 36.607°, 51618 cps respectively). This occurrence was also determined in the formulation of the delayed release system with the addition of GMS to ovalbumin revealing similar θ values for ovalbumin when compared to the optimized system (36.595°, 554709 cps and 36.5828°, 526772 cps for the optimized and delayed release systems respectively). This analysis confirmed that the various components of the Dual-Biotic System had not chemically reacted during the formulation process and that their respective matrices and crystalline structure had been maintained with respect to their native forms. This result is in correlation with the FTIR spectra determined in Section 5.3.2. XRD profiles of the crosslinked ovalbumin system compared to the non-crosslinked ovalbumin as well as the GMS-Amoxicillin combination compared to its native components are shown in Figure 5.13a and 5.13b respectively.

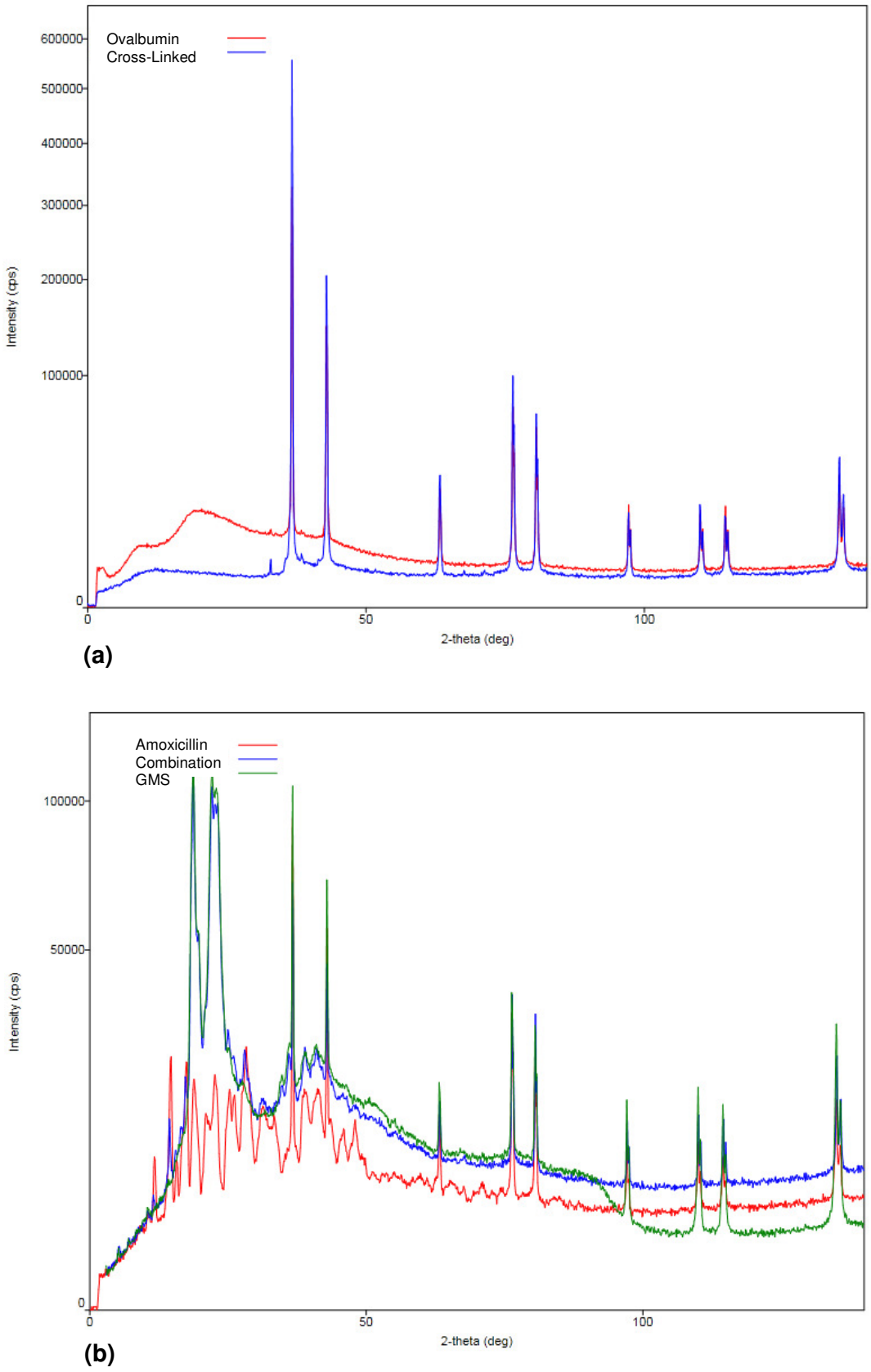


Figure 5.13: XRD profiles of (a) the crosslinked and non-crosslinked ovalbumin systems and (b) the GMS-Amoxicillin combination used in the Dual-Biotic System.

5.3.14 A calibration curve for amoxicillin trihydrate

A calibration curve was constructed for the determination of amoxicillin concentrations present within *in vitro* drug release samples. Known concentrations of amoxicillin were prepared and analyzed at a wavelength of 273nm. Results of the constructed calibration curve for amoxicillin (Figure 5.14) determined an absorbitivity value of 2.4523 with a R^2 value of 0.99. The absorbitivity value was utilized in all *in vitro* drug release evaluations with amoxicillin concentrations calculated utilizing Beer-Lamberts Law.

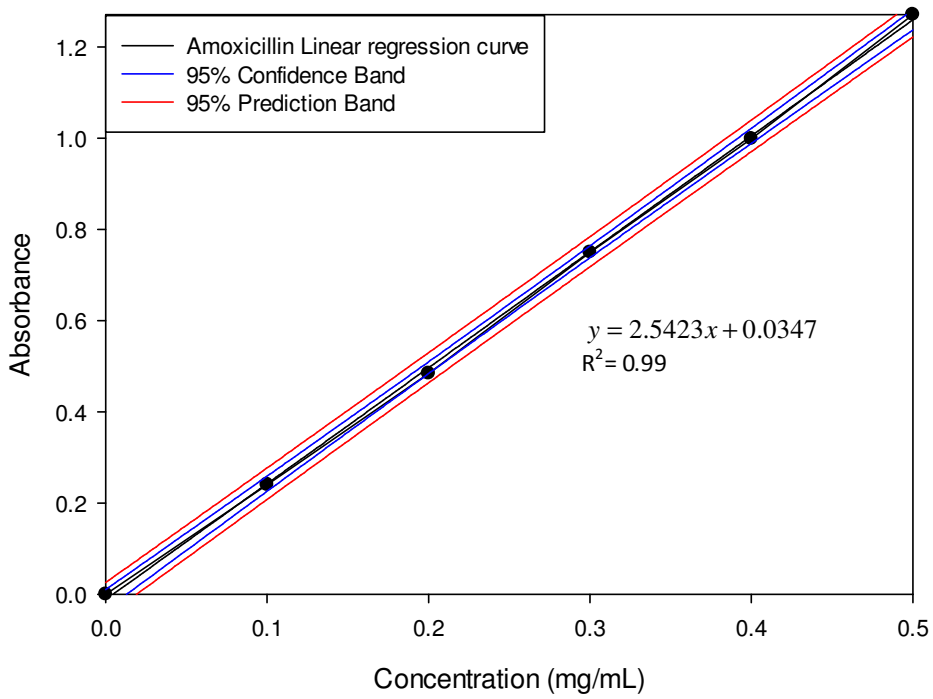


Figure 5.14: Constructed calibration curve for the determination of amoxicillin conducted at a wavelength of 273nm.

5.3.15 *In vitro* release profile of the Dual-Biotic System

The site-specific Dual-Biotic System was formulated utilizing a gastro-resistant polymer coating solution to overcome the issue of varying transit times associated with multi-particulate systems as well as for the prevention of the delayed release system being exposed to gastric conditions prior to delivery to the small intestine. To simulate transition of the delivery system through the gastrointestinal tract, the Dual-Biotic System was subjected to simulated gastric conditions for 2 hours, and thereafter transferred to simulated intestinal conditions for 6 hours. Results of the analysis (Figure 5.15) showed no amoxicillin or *L. acidophilus* release during the simulated gastric conditions analysis with subsequent release determined upon transition to simulated intestinal conditions. This release occurred as previously stated through the delayed exposure of the optimized system to intestinal

conditions and the subsequent release of *L. acidophilus* over a 4 hour period to MPC. The initial lag seen in the FDR and probiotic release determined can be attributed to the time allowed for the dissolution of the gastro-resistant polymer coating in solution. The control of *L. acidophilus* samples analyzed with UV spectroscopy (ε=19.7571 at 208 nm determined in Chapter 3, Section 3.3.12) determined the maximum release of probiotic bacteria within the 4 hour period intended (4-8 hours) with a FDR of less than 0.2 within the delayed release period. This as stated can be attributed to the incomplete encapsulation of the optimized system by GMS. The resultant *L. acidophilus* in dissolution media was, however, not quantifiable by luminescence due to the presence of functional amoxicillin within solution prior to the 2 hour delayed release period (2-4 hours). The release profile of amoxicillin was also evaluated with the rapid dissolution of amoxicillin seen. This could be due to the minimally compacted nature of amoxicillin within the capsule in combination with the delay in release from the gastro-resistant capsule. The rapid hydrolysis by penicillinase was beneficial in this regard due to the inability of amoxicillin to achieve immediate dissolution upon exposure to intestinal conditions. Penicillinase, however, did inactivate the antibiotic prior to the release of *L. acidophilus* proving the effectiveness of the Dual-Biotic System to deliver and protect function probiotic bacteria in conjunction with an antibiotic.

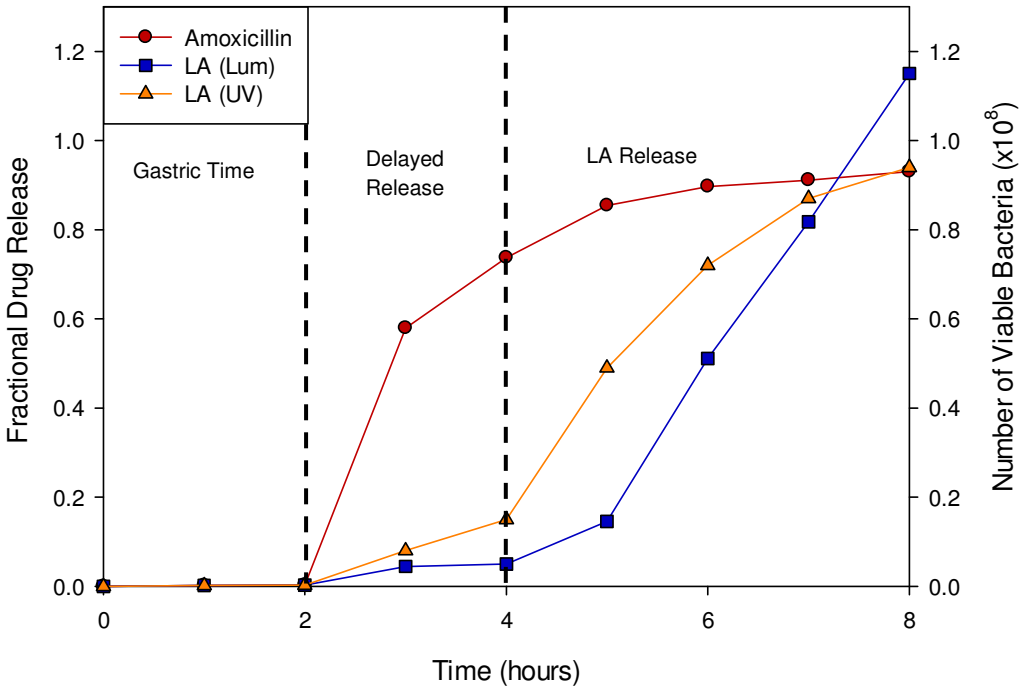


Figure 5.15: Release profile of the site-specific Dual-Biotic System for the concurrent delivery of amoxicillin (determined through UV spectroscopy) and *L. acidophilus* (LA) probiotic (determined through luminescence and UV spectroscopy) (n=3; SD ≤ 0.016 in all cases).

5.3.16 Stability of the optimized, delayed release and Dual-Biotic systems

Stability analysis of the delayed release and Dual-Biotic systems was vital in determining bacterial viability over time. Whilst a moderately high humidity and temperature was utilized in this analysis when compared to other studies, the results obtained can be useful in the determination of the stability enhancing effects of GMS when compared to the optimized system (Silva *et al*., 2013). Samples, before and after conduction of the stability analysis, were also analyzed for moisture content for a suitable correlation to be developed.

Results of the stability analysis conducted on the delayed release system revealed a 15.23% decrease in bacterial cell counts after the three month storage period. Whilst being higher than expected, this result can be attributed to the relative humidity levels employed (Silva *et al*., 2013). The utilized parameters did, however, provide important data on the effect of GMS on the stability of the formulated granules due to its hydrophobic properties. When compared to the delayed release system, the percentage cell count decrease of the optimized system was significantly higher (Table 5.6). This was due to the lack of protection against moisture resulting in greater moisture content after the stability analysis period when compared the delayed release system. The resultant moisture content (11.402%) when compared to the delayed release system (5.802%) explained the result determined (Table 5.7). The increase in moisture content ultimately resulted in the decrease stability of the system and thus the probiotic bacteria over time.

Controls of delayed release system samples refrigerated and kept at ambient room temperature over three months revealed the effect of moisture and temperature on the stability of delayed release granules. This evaluation would provide important information on the stability of the probiotic bacteria under different storage conditions that can be correlated with the probiotic component of the Dual-Biotic System. Refrigerated samples at 4℃ maintained probiotic stability (low percentage decrease of 3.85%) and due to the isolated nature of the analysis, moisture content levels had a minimal increase when compared to the other analyses conducted. Samples at room temperature (25℃), whilst having a lower moisture content than that of the humidifier samples, did not adequately provide stability to the probiotic system, with a significant decrease of 10.38% bacterial cell counts observed. This result therefore confirmed that the stability of the delayed release system was significantly modified by both temperature and humidity but was acceptable due to the high temperature and relative humidity utilized. The instability of *L. acidophilus* itself is also highlighted through this control with the majority of bacterial cell counts being decreased due to probiotic instability.

The Dual-Biotic System was also analyzed to determine formulation stability over time. Whilst the probiotic component of the system would remain relatively similar to the delayed release system, the effect of amoxicillin effectiveness over time was evaluated. Moisture content analysis of the dual-biotic delivery system revealed values similar to that of the delayed release system (6.077%). Differences, whilst minimal, however occurred when the bacterial cell counts were determined. The increased percentage decrease in the bacterial counts (approx. 4%) can be attributed to the exposure of a low fraction of probiotic bacteria to amoxicillin within the hard gelatin capsule. This was in correlation with *in vitro* probiotic release profiles in Section 5.3.15 that noted the immediate release of a small fraction of *L. acidophilus* in solution due to the incomplete coating of the optimized granules by GMS. The stability of the delayed release and Dual-Biotic systems were therefore confirmed in the accelerated study which even at the high temperature and relative humidity levels utilized still maintained probiotic cells counts within an acceptable range to provide functional health benefits. Improvements of the Dual-Biotic System would also prove to be beneficial with desiccants and bacterial stability enhancers such as ascorbic acid, as highlighted in Chapter 2, Section 2.2.2, possibly advantageous to overcome the issue of probiotic instability over time.

Table 5.6: Decrease in bacterial cell counts of the delayed release, optimized and Dual-Biotic systems after conduction of accelerated stability analysis in a humidifier.

	Delayed Release	**Optimized**	**Dual-Biotic**
Percentage decrease (%)	15.23	23.30	18.83

Table 5.7: Moisture content (%) of the delayed release, optimized and Dual-Biotic systems prior to and after conduction of accelerated stability analysis.

System	Humidifier		Refrigeration		Ambient	
	Initial	**3 Months**	**Initial**	**3 Months**	**Initial**	**3 Months**
Delayed Release	1.420	5.802	1.420	2.109	1.420	3.950

	Humidifier	
	Initial	**3 Months**
Optimized	3.051	11.402
Dual-Biotic	1.420	6.077

5.4 Concluding Remarks

The formulated Dual-Biotic System evaluated in this chapter has been successfully proven to protect and delay the release of *L. acidophilus* in simulated gastrointestinal conditions. The use of GMS as an erodible polymer in the delayed release system is warranted due to hydrophobic nature that provides formulation stability as well as formulation protection *in vitro*. The delayed release system has also been proven to successfully prevent the release of *L. acidophilus* at the ratio determined. The Dual-Biotic System was also deemed to be stable in accelerated stability studies performed with a minimal decrease in bacterial cell counts observed. The cell counts determined were, however, of an acceptable level to produce a therapeutically effective probiotic formulation.

CHAPTER 6
EX VIVO ANALYSIS OF THE DUAL-BIOTIC SYSTEM

6.1 Introduction

Intestinal flora supplementation through the use of probiotics products can often be highly variable, dependent on patient factors such as varying gastrointestinal conditions upon oral administration, age and race. Whilst *in vitro* analyses have been shown to be effective in the correlation of results to *in vivo* analysis with many other drug delivery systems, the use of excised animal tissue in the *ex vivo* evaluation of probiotic formulations can provide a more accurate prediction of *in vivo* phenomena at a laboratory level. This can be attributed to the susceptibility of probiotic bacteria to changes in intestinal fluid composition as well as their ability to adhere to intestinal mucosa binding sites.

Bacterial adhesion to intestinal mucosa is vital for intestinal flora to exert its functional health benefits for the host. Intestinal flora naturally have a high bacterial adhesion potential, however, this can be improved on with the addition of adhesion enhancers within growth media such as polyunsaturated fatty acids, examples being α-linolenic acid and arachidonic acid (Kankaanpää *et al*., 2004; Yilmaz-Ersan, 2013). The theoretical basis of this modification is that the greater the hydrophobicity of a bacterial cell, the greater its adhesion potential to intestinal mucosa. Therefore by altering the fatty acid composition of bacteria, one can improve its adhesion potential to ultimately allow for greater functional health benefits to occur (Kankaanpää *et al*., 2004). This is, however, a complex task to undertake. The addition of formulation excipients can also have increased effects on bacterial adhesion with mucoadhesive additives increasing bacterial adhesion to intestinal mucosa (Yadav Nisha *et al*., 2013). With variations between individual probiotic species reported, it is therefore of vital importance that all probiotic formulations are analyzed for their adhesion potential to ensure health benefits for a patient. This chapter therefore analyzed the adhesion potential of the administered *L. acidophilus* probiotic and compared it to a conventional *Lactobacillus* probiotic formulation. With intestinal fluid aspiration being the primary method of bacterial cell quantification during *in vivo* studies, the possibility of decreased intestinal fluid cell counts due to a higher bacterial adhesion to intestinal mucosa exists. This would result in an inaccurate estimation of the protective functionality of either the Dual-Biotic System or conventional formulation warranting the evaluation of the individual probiotic bacterial adhesion properties prior to *in vivo* analysis.

As highlighted in Chapter 2, Section 2.4, the ability of probiotic bacteria to compete against pathogenic bacteria for intestinal mucosa adhesion sites has been widely debated. Modification of an adhesion study can provide more insight into the protective functionality of probiotic bacteria and was therefore also included in this chapter. *E. coli* is a common pathogenic bacterium, which is naturally part of gastrointestinal flora and is known to cause gastrointestinal ailments such as diarrhea and gastroenteritis. *C. difficile*, whilst not being a common constituent of gastrointestinal flora when compared to *E. coli*, does however have a substantial role in gastrointestinal pathology and is of greater importance in this study. *C. difficile* is the regarded as the predominant cause of antibiotic-associated diarrhea, the pathological condition the Dual-Biotic System intends on addressing. Its inclusion in the bacterial adhesion analysis is therefore of vital importance to ensure that therapeutic benefits of the Dual-Biotic System are maintained in the prevention of antibiotic-associated diarrhea.

Intestinal flora have an inert resistance to the effects of bile salts and other potentially destructive constituents of intestinal fluid. This resistance is variable in probiotic bacteria depending on the species of bacteria utilized. *L. acidophilus* is naturally found in the gastrointestinal tract and resultantly possesses a resistance to bile salt degradation. This resistance is applicable, however, to natural strains of *L. acidophilus*. *Ex vivo* release studies in intestinal fluid would therefore be advantageous in ascertaining the survival of the *L. acidophilus* strain utilized upon exposure to intestinal conditions during *in vivo* studies. Other *ex vivo* studies performed on the Dual-Biotic System included in this chapter includes antibiotic penetration studies which will ensure the rapid absorption of amoxicillin trihydrate by intestinal mucosa preventing physical interaction between the probiotic and antibiotic after the two hour delayed release period. This is undertaken utilizing excised intestinal mucosa which provides more accurate results when compared to cellulose membrane.

With the conduction of *ex vivo* studies, the methodology utilized for quantification of bacterial cells in this study (luminescence) would not suffice. This is due to the inability of the luminescence protocol to differentiate between different strains of bacteria. This is significant due to the wide variety of bacterial cells present in intestinal fluid and the different pathogenic bacteria cells utilized in the competitive inhibition studies. Inoculation onto agar plates therefore became a viable option as the different bacterial cells can be selectively visualized through this method. Contamination by other bacterial species not included in the *ex vivo* analyses, primarily in the utilization of aspirated intestinal fluid, was however an issue. For this reason a modified MRS agar was formulated that could selectively grow

Lactobacillus bacteria in porcine intestinal fluid. This agar could therefore be utilized as the quantification media for all *ex vivo* studies, collaborated with luminescence, and further utilized during *in vivo* studies, where aspirated intestinal fluid can be inoculated and increases in *Lactobacilli* cell counts could be determined.

This chapter therefore provides the formulation and analysis of a modified MRS agar for the selective enumeration of *Lactobacilli* in aspirated porcine intestinal fluid that is utilized in both *ex vivo* and *in vivo* analyses. *Ex vivo* analyses conducted include probiotic adhesion evaluations, competitive adhesion analyses and antibiotic penetration studies. These analyses would ultimately prove the effectiveness of the Dual-Biotic System to a greater extent than analyses conducted during *in vitro* analyses and can be furthermore correlated to a commercially available *Lactobacillus* formulation analyzed during *in vivo* analysis.

6.2 Materials and Methods

6.2.1 Materials

Lactobacillus acidophilus ATCC 314, *Bacillus cereus* ATCC 11778, *Candida albicans* ATCC 10231, *Clostridium difficile* ATCC 43953, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27858 and *Staphylococcus aureus* ATCC 25923 swabs were purchased from Davies Diagnostics (Randburg, South Africa). MRS Agar and Tryptone Soya Agar were purchased from Oxoid (Hampshire, UK). Raw egg ovalbumin was obtained from commercial chicken eggs (Nulaid®, Grade 1, Grain Fed) purchased from Pick n Pay® Stores (Johannesburg, Gauteng, South Africa). BacTitre-Glo microbial cell viability assay was purchased from Promega (Madison, WI, USA). Genipin, amoxicillin trihydrate, penicillinase derived from *Bacillus cereus*, nystatin, amphoteracin B, fluorescein isothiocyanate and sulforhodamine B were purchased from Sigma-Aldrich (St Louis, MO, USA). Glyceryl monostereate was purchased from Holpro Analytics (Johannesburg, Gauteng, SA). Excised porcine intestinal mucosa and aspirated gastrointestinal fluid were sourced from Central Animal Services (University of the Witwatersrand, Johannesburg, South Africa). All other chemicals were of analytical grade and used as received.

6.2.2 Determination of the selective growth properties of MRS Agar for the selective enumeration of *Lactobacilli*

MRS agar was prepared as per manufacturer specifications by adding 62g of agar medium to 1L of distilled water and autoclaving at 121℃ for 1 hour. After pouring into sterile agar plates and ascertaining sterility through purity studies, a loop full of *Bacillus cereus*, *Candida*

albicans, *E. coli, L. acidophilus, Pseudomonas aeruginosa* and *Staphylococcus aureus* were inoculated onto the agar and incubated at 37℃ for 48 hours under aerobic conditions (*B. cereus*, *C. albicans*, *E. coli, P. aeruginosa* and *S. aureus* only) and anaerobic conditions for *B. cereus*, *C. albicans*, *E. coli*, *L. acidophilus*, *P. aeruginosa* and *S. aureus*. Whilst anaerobic incubation conditions would prevent the growth of *B. cereus*, *P. aeruginosa* and *S. aureus*, it was necessary to perform aerobic incubation of the cultures as an accurate depiction of the selective growth properties of MRS media could be attained. *C. albicans* and *E. coli* are able to grow in both aerobic and anaerobic conditions. *B. cereus*, *C. albicans*, *E. coli*, *P. aeruginosa* and *S. aureus* were cultured from lyophilized bacterial stocks inoculated into sterile Tryptone Soya Broth for 24 hours. Controls of uninoculated MRS agar plates and TSA agar plates inoculated with *B. cereus*, *C. albicans*, *E. coli, P. aeruginosa* and *S. aureus* were included to ensure purity of the MRS agar and viability of the cultures respectively. Bacterial growth was visually determined after incubation for the MRS agar and control groups.

6.2.2.1 *Preparation of a modified MRS Agar for the selective enumeration of Lactobacillus in aspirated porcine intestinal fluid*

The MRS agar was formulated and sterilized as outlined in Section 6.2.2. Once allowed to cool to a molten state (70℃), 5mL/L of 1M acetic acid was added to lower the pH of the agar medium to inhibit the growth of *E. coli*. The molten agar was thereafter poured into sterile agar plates and allowed to cool to room temperature. After conduction of purity studies, *B. cereus*, *C. albicans*, *E. coli*, *L. acidophilus*, *P. aeruginosa* and *S. aureus* were inoculated onto the modified acetic acid-containing MRS agar and incubated as outlined in Section 6.2.2. Bacterial growth was visually determined after incubation at 37℃ for 48 hours under anaerobic conditions.

6.2.2.2 *Selection of the antifungal to be incorporated into the modified MRS agar*

The modified MRS agar was prepared as outlined in Section 6.2.2.1 with the addition of acetic acid. Amphoteracin B and nystatin were thereafter added to individual batches of agar medium to ascertain their inhibitory effects on *C. albicans* and therefore determine which would be included in the *Lactobacillus*-specific agar. Amphoteracin B was prepared by dissolving 10mg in 10mL Dimethyl sulfoxide (DMSO). Serial dilutions were prepared to determine the concentration of Amphoteracin B required for the inhibition of *C. albicans* on the modified MRS agar. Samples of 100µL at concentrations of 1mg/mL, 0.1mg/mL and 0.01mg/mL amphoteracin B was added to 20mL of molten modified MRS agar (at 70℃) and allowed to cool. Thereafter *C. albicans* was inoculated onto the agar media and incubated at 37℃ for 48 hours under anaerobic conditions. Nystatin was prepared by adding 10mg

nystatin to 10mL DMSO with a series of dilutions prepared to determine concentration of nystatin required for *C. albicans* inhibition. Samples of 100µL at a concentration of 1mg/mL, 0.1mg/mL and 0.01mg/mL of nystatin was added to 20mL of molten modified MRS agar media and allowed to cool. Thereafter *C. albicans* was inoculated onto the agar media and incubated as outlined.

6.2.2.3 *Formulation and microbial analysis on the effectiveness of the Lactobacillus-specific agar*

The effectiveness of the *Lactobacillus*-specific agar was determined by inoculating *B. cereus*, *C. albicans*, *E. coli*, *L. acidophilus*, *P. aeruginosa* and *S. aureus* onto the modified MRS agar containing 5mL/L acetic acid and 5mg/L nystatin solution. Controls were included as outlined in Section 6.2.2. Purity studies were conducted as previously outlined in Chapter 3, Section 3.2.2.

6.2.3 Bacterial adhesion analysis utilizing excised porcine intestinal mucosa

The adhesion properties of the delivered *L. acidophilus* probiotic bacteria was determined using the *ex vivo* bacterial assay method outlined by Kristiansen *et al*. (2011) with modifications. This analysis was vital in determining the bacterial adhesion properties of *L. acidophilus* to ensure functional health benefits. The analysis will also correlate with results determined during *in vivo* studies where bacteria would adhere to intestinal mucosa upon release from the Dual-Biotic System. Briefly, the excised pig intestine was washed with sterile saline, placed into sterile saline solution containing viable *L. acidophilus* bacteria and allowed to rest for 1 hour prior to washing of the mucosa to remove residual bacteria on the mucosal surface. The intestinal mucosal surface was thereafter scraped off using a sterile scalpel (Figure 6.1), placed in 10mL sterile saline, vortex mixed for 5 minutes and the solution streaked onto the formulated *Lactobacillus*-specific agar in Section 6.2.2.3. The agar plates were thereafter incubated under anaerobic conditions at 37℃ for 48 hours. As a control, intestinal tissue not placed in bacterial solution was included. The above procedure was repeated utilizing a conventional *Lactobacillus* formulation to correlate *L. acidophilus* adhesion to probiotics containing *Lactobacilli* bacteria presently on the market. As the conventional formulation would also be analyzed during *in vivo* analysis, this analysis would determine the accuracy of results determined through intestinal fluid aspiration. The bacterial adhesion studies were conducted in triplicate for accuracy of results.

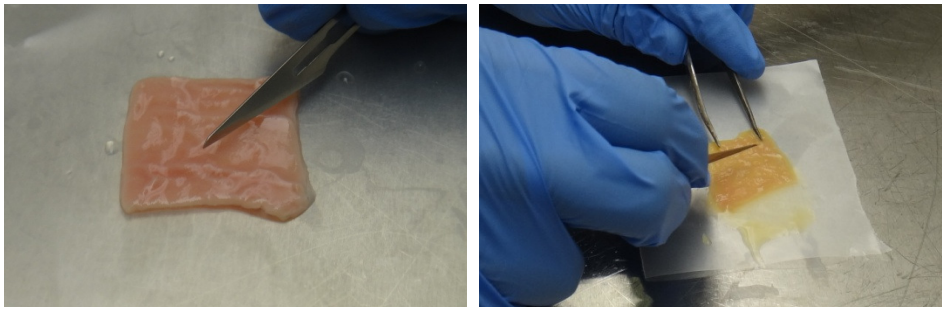


Figure 6.1: Digital images of the removal of the porcine intestinal wall mucosa.

6.2.4 Analysis of the competitive bacterial inhibition properties of *L. acidophilus*

The protective functionality of the delivered *L. acidophilus* bacteria was determined utilizing a modified method as stated in Section 6.2.3. The excised intestinal tissue was washed with sterile saline and placed in a solution of amoxicillin for 15 minutes to simulate destruction of the intestinal flora by the delivered antibiotic. Thereafter the intestinal mucosa was placed in sterile saline containing penicillinase for 30 minutes to inactivate residual amoxicillin present on the tissue wall. The intestinal tissue was then washed with sterile saline and placed in solution of *L. acidophilus* for 1 hour to promote adhesion of the delivered *L. acidophilus* with the residual bacteria thereafter removed. The excised mucosa was thereafter placed in a solution of *E. coli* for 30 minutes, the mucosa washed and the surface removed as stated in Section 6.2.3. The removed surface, after being placed in sterile saline and vortex mixed, was inoculated onto *Lactobacillus*-specific agar for *L. acidophilus* and TSA for *E. coli* with the protective functionality of *L. acidophilus* determined by counting the CFUs after incubation at 37℃ for 48 hours under anaerobic and aerobic conditions respectively. Whilst traditional MRS media mildly supports the growth of *E. coli*, TSA was utilized as the growth media due to the large CFUs formed on the agar surface after incubation.

The protective functionality assay was repeated as outlined utilizing *Clostridium difficile* as the pathogenic bacterium. *C. difficile* is the most common cause of antibiotic-associated diarrhea and its competitive inhibition by *L. acidophilus* was vital in the prevention of the antibiotic side-effect the Dual-Biotic System was designed to overcome. *C. difficile* (100µL) was placed in the respective solution with the mucosa thereafter removed. After being vortex mixed, streaked onto Thioglycolate agar and incubated under anaerobic conditions utilizing the anaerobic gas pack method, the CFUs after incubation was counted. Controls of uninoculated media were placed in all analyses with the respective protective functionality assays conducted in triplicate for accuracy of results.

6.2.4.1 *Isolation and stock maintenance of E. coli from lyophilized bacterial stocks*

E. coli cells were cultured from lyophilized bacterial stocks by inoculation into sterile Tryptone Soya Broth and incubating at 37℃ for 48 hours under aerobic conditions. Sterility of the Tryptone Soya Broth was confirmed utilizing the purity analysis highlighted.

6.2.4.2 *Quantification of cultured E. coli*

Quantification of *E. coli* present in the cultured tryptone soya broth media was undertaken through serial dilution with the respective dilutions transferred onto TSA and incubated at 37℃ for 48 hours. CFU counts were performed on all dilutions after incubation with the number of viable *E. coli* cells in the stock solution determined through calculation.

6.2.4.3 *Isolation and stock maintenance of C. difficile from lyophilized bacterial stocks*

C. difficile cells were cultured from lyophilized bacterial stocks by inoculation into sterile Thioglycolate broth and incubating at 37℃ for 48 hours under anaerobic conditions utilizing the gas pack technique highlighted in Chapter 3, Section 3.2.2.1. *C. difficile* is an obligate anaerobe which requires an oxygen free environment to grow. Therefore the gas pack technique, which produces such an oxygen free chamber, was utilized as opposed to the candle jar technique. Sterility of the thioglycolate broth was confirmed utilizing the purity analysis stated in Chapter 3, Section 3.2.2.

6.2.4.4 *Quantification of cultured C. difficile*

Quantification of *C. difficile* present in the cultured thioglycolate media was determined through serial dilution as stated in Chapter 3, Section 3.2.2.1 with the respective dilutions inoculated onto thioglycolate agar and incubated at 37℃ for 48 hours under anaerobic conditions utilizing the anaerobic gas pack technique.

6.2.4.5 *High-speed fluorescence imaging of porcine intestinal mucosa exposed to L. acidophilus and pathogenic bacteria*

A high-speed fiber-optic fluorescence microscope (Cellvizio® LAB, coupled with Microprobes and ImageCell™ Software; Visualsonics and Mauna Kea Technologies, USA) was employed for *ex vivo* imaging of the porcine intestinal mucosa utilized in the competitive inhibition analysis. Fluorescence labeling of the respective bacterial cells was achieved utilizing the method as outlined by Ejekwumadu and Sarker (2013) with modifications. For the labeling of *L. acidophilus*, bacterial culture that was incubated overnight was centrifuged at 3000rpm for 10 minutes, with the supernatant discarded. The pellet was thereafter suspended in

Fluorescein Isothiocyanate at a concentration of 2µg/mL for a period of 1 hour in an orbital shaker at 37℃. After incubation, the pellet was centrifuged at 3000rpm for a further 10 minutes with the pellet repeatedly washed with sterile distilled water and centrifuged until the pellet had become colorless. *C. difficile* and *E. coli* were labeled with Sulforhodamine B at a concentration of 2µg/mL and isolated as stated. Sulforhodamine B was utilized as a contrast to Fluorescein Isothiocyanate. The competitive inhibition assay was conducted as stated in Section 6.2.4 with fluorescence imaging of the conjugated bacteria undertaken on the porcine intestinal mucosa utilizing the Cellvizio® LAB apparatus.

6.2.5 *Ex vivo* release studies of the Dual-Biotic System in aspirated porcine gastro-intestinal fluid

Ex vivo release studies was performed on the Dual-Biotic System utilizing gastric and intestinal fluid aspirated from the porcine stomach and intestine respectively. Whilst the gastro-resistant coating solution encapsulating the Dual-Biotic System would prevent release of the Dual-Biotic System in aspirated gastric fluid, analysis in aspirated intestinal fluid would accurately prove the effectiveness of the Dual-Biotic System which cannot be perfectly simulated *in vitro*. This is due to intestinal fluid consisting of various constituents that cannot be accurately simulated *in vitro*. These include bile salt concentrations, digestive enzyme concentrations and microbial content. For this reason, the formulated Dual-Biotic System was placed in 20mL of aspirated gastric fluid for 2 hours and thereafter in 20mL of aspirated intestinal fluid for 6 hours. All analyses were conducted in an orbital shaker bath rotating at 25rpm.

6.2.6 Amoxicillin penetration studies across excised pig intestine utilizing a Franz-Diffusion apparatus

Ex vivo penetration studies were conducted on the delivered amoxicillin trihydrate using a Franz-Diffusion (FD) apparatus equipped with a 12mL receptor compartment, clamp and stirrer-bar (Figure 6.2). This was undertaken to ensure rapid amoxicillin permeation across the intestinal mucosa prior to the conduction of *in vivo* studies. Freshly excised intestinal tissue obtained from a euthanized Large White pig was placed between the donor and receptor compartments of the FD apparatus. Simulated plasma (PBS at pH 7.4; 10mL) was used in the receptor compartment. A known concentration of amoxicillin trihydrate (2.5mg/mL) was placed in the donor compartment and permeation determined through analysis of amoxicillin concentration in the receptor compartment over a 6 hour period. All samples were analyzed via UV spectroscopy and the concentration of amoxicillin trihydrate determined through calculation utilizing the calibration curve constructed in Chapter 5,

Section 5.3.14. The flux ($mg.cm^{-2}.hr^{-1}$) of amoxicillin trihydrate across the intestinal mucosa was calculated per unit area at steady-state concentration by linear regression analysis of permeation data utilizing Equation 6.1.

$$J_s = \frac{Q_r}{A \times t} \qquad \text{Equation 6.1}$$

Where:

J_s is the flux,

Q_r is the quantity of amoxicillin trihydrate that diffused through the intestinal mucosa into the receptor compartment (mg),

A is the effective cross-sectional area available for drug permeation (cm^2) and

t is the time of drug exposure to the intestinal mucosa (hours).

The permeability coefficient (*P*), following application of Ficks's law, can be calculated as follows utilizing Equation 6.2:

$$P = \frac{J_s}{C_{donor}} \qquad \text{Equation 6.2}$$

Where:

P is the permeability coefficient,

J_s is the flux and

C_{donor} is the concentration in the donor compartment.

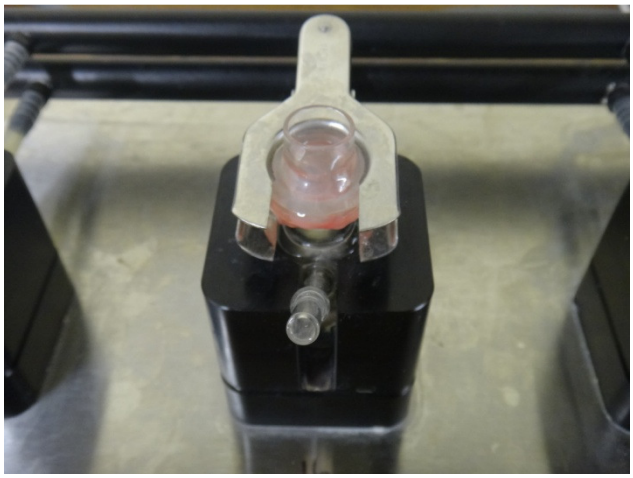


Figure 6.2: Digital Image of the Franz-Diffusion apparatus setup utilized for the antibiotic penetration analysis.

6.2.6.1 *Integrity analysis of the intestinal mucosa*

Integrity analysis of the intestinal mucosa utilized during the antibiotic permeation study was undertaken using a SevenMulti S40 pH/electrical conductivity meter (Mettler-Toledo, Zurich, Switzerland) both prior to and after conduction of the experimental analysis. This was necessary due to compromised biological tissue providing false permeation results. Intestinal integrity was therefore undertaken through the determination of ionic conductivity and calculation of the Resistance Reduction Factor (RF) or damage ratio as per Equation 6.3.

$$RF = \frac{R_0}{R_t} \qquad \text{Equation 6.3}$$

Where:

R_0/R_t is the ratio of the initial mucosal resistance to the mucosal resistance after time t.

6.3 Results and Discussion

6.3.1 Evaluation of the selective growth properties of traditional and modified MRS agar media

Analysis of the growth properties of MRS agar prior to the addition of acetic acid revealed the inhibition of *B. cereus*, *P. aeruginosa* and *S. aureus* under both aerobic and anaerobic conditions (Table 6.1). Minimal growth of *E. coli* was determined with extensive growth of *C. albicans* and *L. acidophilus* ascertained after the incubation period. This collaborates with previous work which determined the growth of clinical strains of *E. coli* on MRS agar. The reason for the selection of the individual species of microbes is as follows: *E. coli* was utilized as the Gram-negative anaerobic bacteria and is furthermore naturally found in the human colon. *S. aureus* and *B. cereus* are both Gram-positive aerobic bacteria and are also found naturally in the human GIT. *C. albicans* is, however, not naturally found in the human intestine or colon but is widely regarded as the reference microbe in respect to other species of yeasts when considering growth or inhibitory media. Controls conducted by inoculating *B. cereus*, *C. albicans*, *E. coli*, *P. aeruginosa* and *S. aureus* onto TSA revealed the viability of the microbes prior to the conduction of the microbial analysis. Inoculation of *L. acidophilus* onto Thioglycolate agar ascertained microbial growth ensuring cell viability with inoculation onto TSA ensuring purity of the culture.

Table 6.1: Growth of the various species of microorganisms.

Microorganism	MRS Agar (aerobic)	MRS Agar (anaerobic)	Tryptone Soya Agar	Thioglycolate Agar
B. cereus	Negative	Negative	Positive	N/A
C. albicans	Positive	Positive	Positive	N/A
E. coli	Positive	Positive	Positive	N/A
L. acidophilus	Positive	Positive	Negative	Positive
P. aeruginosa	Negative	Negative	Positive	N/A
S. aureus	Negative	Negative	Positive	N/A

N/A = Not applicable as not a relevant media for culturing.

Due to this result, modification of the MRS media was required to inhibit the growth of *E. coli* and *C. albicans*. The first step undertaken was to lower the pH of the media utilizing acetic acid. The theoretical basis of lowering the pH of the agar media was to place the media conditions within a range optimal for *L. acidophilus* growth but prevented the growth of other species of bacteria and yeasts. *E. coli*, which is naturally found in the human colon (pH 7.4), would be most susceptible to such a pH decrease. The pH measurements revealed the agar media had decreased from a pH of 5.83 to 5.07 after the addition of acetic acid. This decrease was satisfactory as it inhibited the growth of *E. coli* but promoted the growth of *C. albicans* (Table 6.2). An antifungal would therefore have to be incorporated into the modified MRS agar for the formulation of the *Lactobacillus*-specific agar.

Table 6.2: Growth of the various species of micro-organisms exposed to modified MRS agar containing acetic acid.

Micro-organism	M.R.S Agar + Acetic Acid
B. cereus	Negative
C. albicans	Positive
E. coli	Negative
L. acidophilus	Positive
P.aeruginosa	Negative
S. aureus	Negative

6.3.2 Evaluation of the addition of amphoteracin B to the modified MRS agar

Amphoteracin B, a broad spectrum anti-fungal, was evaluated for the prevention of growth of *C. albicans* and possibly other fungi or yeasts that may be present in intestinal fluid. Results of the evaluation of amphoteracin B showed positive growth for *C. albicans* at all concentrations tested (Table 6.3). A control added to determine if the agar media was contaminated prior to inoculation determined no growth after incubation removing the possibility of contamination.

At the concentrations tested the amphoteracin B would have prevented growth of *C. albicans*. This was confirmed through the repetition of the assay utilizing TSA as opposed to modified MRS agar. Results of this analysis revealed the inhibition of microbial growth after the incubation period. A possible reason for this result is that amphoteracin B was structurally modified upon addition to MRS media, resulting in its ineffectiveness against *C. albicans*. From this analysis, amphoteracin B was proven not be effective in the inhibition of *C. albicans* when added to the modified MRS agar and was therefore not considered for incorporation into the *Lactobacillus*-specific agar.

Table 6.3: Results of the inhibitory effects of amphoteracin B on *C. albicans* when inoculated onto the modified MRS agar and TSA.

Concentration	MRS	Tryptone Soya Agar
1mg/mL	Positive	Negative
0.1mg/mL	Positive	Negative
0.01mg/mL	Positive	Negative
Control	Negative	Negative

6.3.3 Evaluation of the addition of nystatin to the modified MRS agar

With amphoteracin B proving ineffective for *C. albicans* growth inhibition, nystatin, another broad spectrum antifungal was analyzed for incorporation into the modified MRS agar. Preliminary studies utilizing a nystatin anti-microbial disc placed onto inoculated *C. albicans* on the modified MRS agar surface determined the effectiveness of nystatin to inhibit *C. albicans* growth. An inhibitory concentration assay was again performed for nystatin against *C. albicans* with the results determining that 0.1mg/mL nystatin was required for *C. albicans* inhibition (Table 6.4). A control of uninoculated agar revealed no growth depicting purity of the agar medium.

Table 6.4: Results of the inhibitory effects of nystatin on *C. albicans* inoculated onto modified MRS agar.

Concentration	Result
1mg/mL	Negative
0.1mg/mL	Negative
0.01mg/mL	Positive
Control	Negative

6.3.4 Evaluation of the *Lactobacillus*-specific agar for the selective enumeration of *Lactobacilli* in intestinal fluid

A final evaluation was conducted to determine if the formulated *Lactobacillus*-specific agar was specific for *Lactobacilli* bacteria. As before, various categories of bacteria commonly used for determination of microbial inhibition were inoculated onto the *Lactobacillus*-specific agar surface. Results of this analysis revealed the selective enumeration of *L. acidophilus*. Controls of the inoculation of aspirated intestinal fluid revealed single colony types depicting *Lactobacilli* growth. A control of uninoculated agar revealed no growth depicting purity of the agar medium. The results of this evaluation are listed in Table 6.5. Digital images of the growth of *C. albicans*, *E. coli* and *L. acidophilus* inoculated onto the unmodified and modified MRS agar are depicted in Figure 6.3.

Table 6.5: Results of growth of various species of micro-organisms inoculated on the *Lactobacillus*-specific agar.

Microorganism	Result
B. cereus	Negative
C. albicans	Negative
E. coli	Negative
P.aeruginosa	Negative
S. aureus	Negative
L. acidophilus	Positive
Intestinal Fluid	*Lactobacilli* growth
Control	Negative

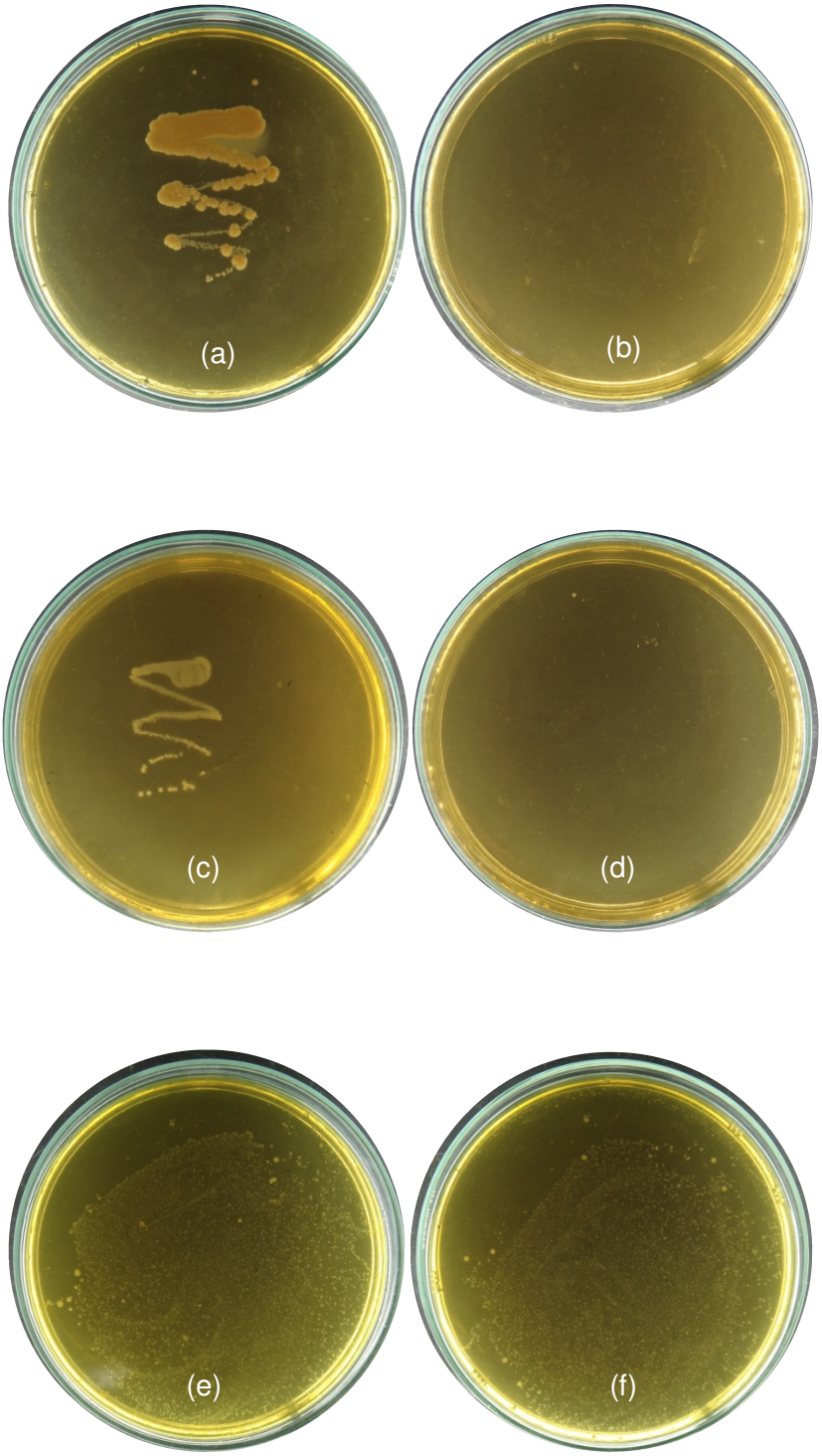


Figure 6.3: Digital Images of the unmodified and *Lactobacillus*-specific MRS agar plates inoculated with (a -b) *C. albicans*, (c-d) *E. coli* and (e-f) *L. acidophilus.*

6.3.5 Evaluation of the bacterial adhesion properties of *L. acidophilus*

Adhesion analyses conducted on *L. acidophilus* revealed the high adhesion potential of the utilized probiotic bacteria. Through calculation, it was determined that *L. acidophilus* occupied the excised intestinal mucosal walls at a concentration of approximately 4000

cells/cm^2 (Table 6.6). This was correlated to the conventional probiotic formulation which was determined to have an adhesion concentration of approximately 4400 cells/cm^2. The similarity between the dual-biotic probiotic species and the conventional system can be attributed to both formulations containing *Lactobacilli* as the active probiotic. Whilst a minor difference can be determined between different species of bacteria, this was considered not to be of any significant importance when utilized in the Dual-Biotic System and the conventional formulation. From this analysis it was revealed that *L. acidophilus* species utilized in the Dual-Biotic System had a high adhesion potential to provide functional health benefits to a patient, but more importantly could be correlated with the analyzed conventional formulation. This result therefore confirmed that intestinal fluid aspiration can be a viable option for the determination of probiotic release from the Dual-Biotic System.

Table 6.6: The adhesion potential of the dual-biotic and conventional systems.

System	Cell Count	Adhesion (cells/cm^2)
Dual-biotic	36000	4000
Conventional	40000	4444

6.3.6 Quantification of the cultured *E. coli* and *C. difficile* cells

Prior to the conduction of the competitive inhibition assay, it was necessary to determine the number of viable pathogenic bacteria present within the stock solutions. This was to create an accurate correlation with *in vivo* conditions, but also due to the phenomenon that a large variation in pathogenic cells opposed to intestinal flora would result in an imbalance in bacterial concentration resulting in the forced removal of intestinal flora intestinal wall binding sites. This was highlighted by Lee *et al*. (2000) where intestinal wall binding by bacterial cells was correlated with the maintenance of microbial equilibrium where a large change in flora composition results in the detachment of bacterial cells from the binding sites.

Results of the quantification analyses determined that the concentration of *E. coli* present in the stock solution was approximately 2.07 x 10^8 viable bacterial cells/mL with *C. difficile* having a concentration of 1.12 x 10^8 viable bacterial cells/mL. Through this analysis, the number of pathogenic bacteria was thus calculated for utilization in the protective adhesion analysis performed.

6.3.7 The protective adhesion properties of *Lactobacillus* probiotic against *E. coli* and *C. difficile*

The protective functionality of probiotic bacteria to inhibit pathogenic bacteria adhesion to intestinal mucosal wall is vital in ensuring gastrointestinal health of a patient. To simulate the probiotic delivery from the Dual-Biotic System, amoxicillin was utilized to remove residual intestinal flora present on the mucosal surface prior to the analysis. This would simulate intestinal flora destruction that occurs through antibiotic intake. Adhesion determination at this point of the protective adhesion assay revealed no CFUs after incubation revealing that amoxicillin had removed all viable bacteria on the intestinal mucosal wall (Table 6.7). The intestinal mucosa was thereafter exposed to probiotic bacteria from the dual-biotic and conventional systems where bacterial adhesion took place as determined in Section 6.3.5.

Once probiotic bacterial adhesion had occurred, the intestinal mucosa was placed in individual pathogenic bacteria solutions of *E. coli* and *C. difficile*. Results of the dual-biotic bacteria analysis revealed a significant decrease in the adhesion of the pathogenic bacteria (approximately 1089 and 222/cm^2 for *E. coli* and *C. difficile* respectively) when compared to intestinal mucosa exposed to the pathogenic bacteria without the adhesion of probiotic bacteria (approximately 2111 and 1900/cm^2 respectively). This decrease was furthermore determined with regards to the conventional system with a decrease of in pathogenic bacteria occurring (2111 to 1033 and 1900 to 244/cm^2 for *E. coli* and *C. difficile* respectively). While minor differences between the two systems were observed, the relatively similar bacterial adhesion confirmed that the strains of *Lactobacilli* utilized in the dual-biotic and conventional systems could be quantified through intestinal fluid aspiration. This result was significant as the removal of intestinal wall from the pig model and quantification through the bacterial adhesion assay would not be feasible due to the time points utilized. For this reason, intestinal fluid aspiration would be a viable option where CFUs in the intestinal fluid can be correlated with bacterial adhesion when comparing the Dual-Biotic System to the conventional system. Probiotic release profiles can therefore be calculated through this sampling technique with the *Lactobacillus*-specific agar utilized for selective enumeration of the probiotic bacteria.

Table 6.7: Bacterial adhesion of *Lactobacillus* probiotic (*LP*), *E. coli* and *C. difficile* on excised intestinal porcine mucosa.

System	*LP* Cell Count	*LP* Adhesion (cells/cm^2)	*E. coli* Cell Count	*E. coli* Adhesion (cells/cm^2)	*C. difficile* Cell Count	*C. difficile* Adhesion (cells/cm^2)
Dual-biotic	32000	3556	9800	1089	N/A	N/A
Dual-biotic	32000	3556	N/A	N/A	2000	222
Conventional	33000	3667	9300	1033	N/A	N/A
Conventional	31000	3444	N/A	N/A	2200	244
E. coli	N/A	N/A	19000	2111	N/A	N/A
C. difficile	N/A	N/A	N/A	N/A	17000	1889

N/A = not applicable to analysis

6.3.8 Fluorescence imaging of conjugated bacterial cells adhered to porcine intestinal mucosa

Fluorescence imaging of the porcine intestinal mucosa after conduction of the competitive inhibition assay revealed the effectiveness of *L. acidophilus* to restrict the adherence of the respective pathogenic bacteria cells to the intestinal wall. Figure 6.4 depicts the high adherence potential of *L. acidophilus* to the intestinal wall where an abundance in flourescein-conjugated cells adhered to the mucosal surface was noted. Upon exposure to the respective pathogenic bacteria, the flourescein-conjugated cells maintained their high adherence potential with minimal adherence of sulforhodamine B-labeled pathogenic bacteria visualized (Figure 6.4a-b). It was therefore visually confirmed that the probiotic *L. acidophilus* cells incorporated into the Dual-Biotic System adhered well to porcine intestinal mucosa and upon exposure to common pathogenic bacteria, competitively inhibited cellular adhesion. This result was in correlation with the microbiological analysis conducted in Section 6.3.7 where minimal pathogenic bacteria adhesion was determined.

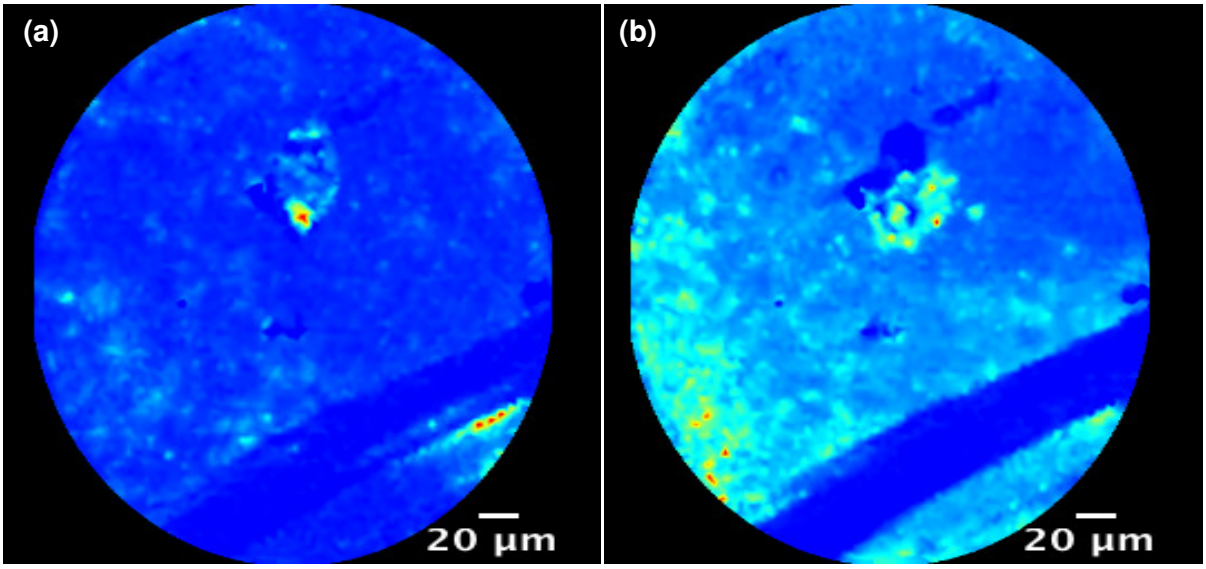


Figure 6.4: Fluorescence images of *L. acidophilus* cells (light blue) adhered to porcine intestinal mucosa exposed to (a) *C. difficile* and (b) *E. coli* in orange.

6.3.9 Probiotic release profiles of the Dual-Biotic System utilizing aspirated porcine intestinal and gastric fluid

Release profiles of the Dual-Biotic System revealed no increase in microbial cell counts within aspirated gastric fluid (Figure 6.5). This determined that the gastro-resistant capsule utilized had remained intact when exposed to porcine gastric fluid warranting its use *in vivo*. Upon transition to aspirated intestinal fluid, the Dual-Biotic System released in a similar manner to that seen during *in vitro* studies with the notable exceptions being a greater increase in microbial cell count after 6 hours in the aspirated intestinal fluid. This is attributed to the increase in bacterial concentrations due to intestinal fluid providing better growth conditions compared to *in vitro* dissolution media. From this analysis, it was determined that the Dual-Biotic System would successfully release amoxicillin trihydrate and *L. acidophilus* in a manner consistent with *in vitro* studies conducted. This ultimately ensured that the formulated Dual-Biotic System would theoretically release in the intended manner during *in vivo* analysis.

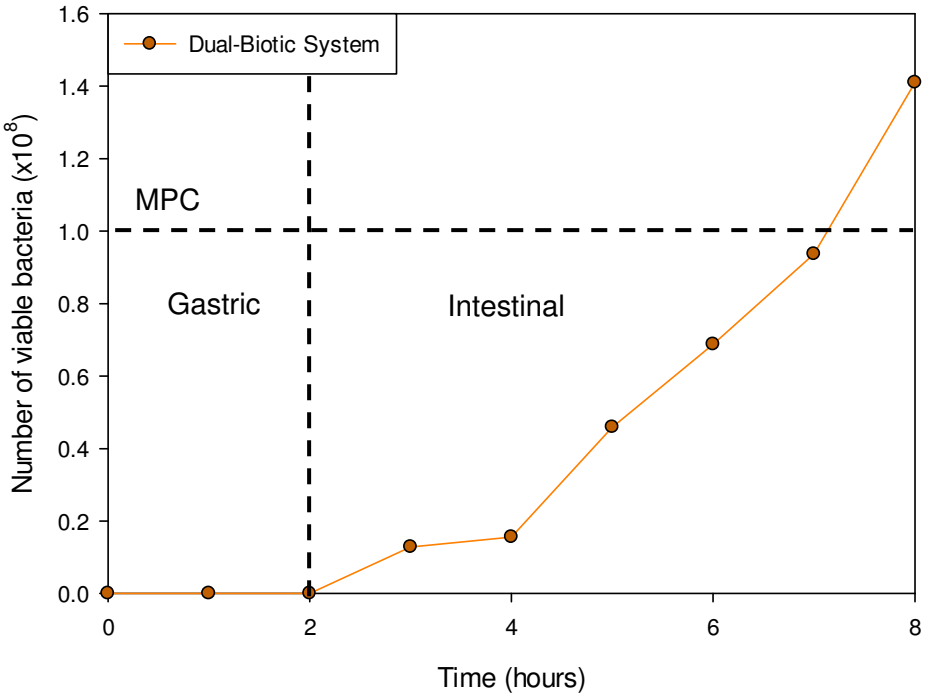


Figure 6.5: Probiotic release profile of the Dual-Biotic System in aspirated porcine gastric (0-2) and intestinal fluid (2-8) (n=3; SD≤ 0.028 in all cases).

6.3.10 Amoxicillin permeation analysis

Permeability analysis of amoxicillin trihydrate revealed a constant increase in the rate of flux over a period of 3 hours to steady-state concentration (Figure 6.6). At this point saturation of the transport mechanism of amoxicillin trihydrate across the intestinal mucosa had occurred where no further molecules entered the receiver compartment of the FD apparatus. This analysis depicted the increased permeability of amoxicillin trihydrate which *in vivo* has been shown to reach T_{max} at 1.28 hours. This property can be attributed to the hydrophilic properties of amoxicillin trihydrate leading to greater dissolution in addition to the rapid absorption associated with oral delivery of amoxicillin. The flux at steady-state was determined to be 0.6123mg.cm^{-2}.hr^{-1} with the permeability coefficient determined to be 1.1597 (Table 6.8). These parameters determined that in the pig model, amoxicillin trihydrate would be rapidly absorbed in line with the pharmacokinetic properties of the drug.

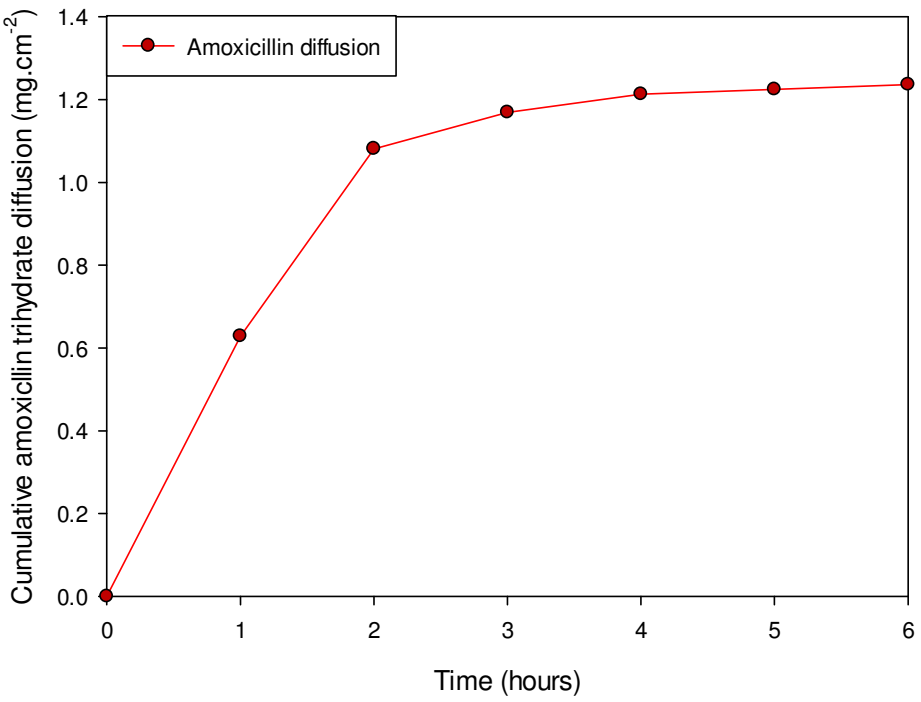


Figure 6.6: *Ex vivo* permeation profile of amoxicillin trihydrate across excised porcine intestinal mucosa (n=3; SD≤ 0.04 in all cases).

Table 6.8: Amoxicillin flux and permeability co-efficient as determined through *ex vivo* permeation analysis.

Drug	Flux (Js; $mg.cm^{-2}.hr^{-1}$)	Permeability Co-efficient
Amoxicillin trihydrate	0.6123 ± 0.006	1.1597 ± 0.011

6.3.11 Porcine intestinal mucosa integrity evaluation

Evaluation of the porcine intestinal mucosa determined a RF of 1.545. This analysis therefore confirmed that the intestinal mucosa had maintained its integrity with a minimal variation in resistance factor despite its fragile nature. The RF calculated therefore validates the amoxicillin permeation results determined.

6.4 Concluding Remarks

Through *ex vivo* analysis, the Dual-Biotic System was proven to be effective in the protection and release of probiotic *L. acidophilus*. Permeation analysis of amoxicillin trihydrate revealed the rapid movement of the antibiotic across intestinal mucosa correlating with the pharmacokinetic properties of the drug. With adhesion studies performed, *L. acidophilus* was proven to effectively adhere to intestinal mucosal binding sites and when exposed to foreign

pathogenic bacteria, resisted detachment and thus prevented bacterial adhesion. This adherence was in a similar fashion to the conventional system analyzed revealing that a comparison between the dual-biotic and conventional formulation can be completed utilizing aspirated intestinal fluid. Furthermore MRS agar was successfully modified to ensure the selective enumeration of *Lactobacilli* in aspirated intestinal fluid. The formulated Dual-Biotic System was therefore advanced to *in vivo* analysis where the *Lactobacillus*-specific agar was utilized for the quantification of *Lactobacilli* in aspirated intestinal fluid.

CHAPTER 7
IN VIVO EVALUATION OF THE DUAL-BIOTIC SYSTEM IN THE LARGE WHITE PIG MODEL

7.1 Introduction

**Ethical clearance for this in vivo investigation was obtained from the Animal Ethics Screening Committee of the University of the Witwatersrand. Clearance Number: AESC 2012/26/05 (Appendix D).*

In vivo analysis of pharmaceutical delivery systems provide vital information on the release and absorption characteristics that cannot be accurately simulated *in vitro* and *ex vivo*. With respect to probiotic systems, physiological barriers to functional bacterial delivery such as gastric acid degradation, gastrointestinal secretions, residual gastrointestinal microbiota and gastrointestinal contents are of significant importance with *in vivo* analysis being the only accurate way of accounting for these phenomena. The selection of an ideal animal model that would correlate well with human patients was therefore undertaken. Large animals, such as monkeys, dogs and pigs, due to their size and physiological and anatomical similarities with humans are considered the most ideal models for *in vivo* analysis of oral pharmaceutical delivery systems. In addition, these models have a similar gastrointestinal microbial environment to human subjects (Mare, 2009). The use of monkeys and dogs are often complicated, with the economic feasibility often questioned in addition to the ethical implications of such a study. Pigs, however, have been widely utilized in *in vivo* studies involving novel drug delivery systems (Sjögren *et al*., 2014). This is due to the physiological similarities of pigs with humans such as its nearly identical Cytochrome P450 system, the primary enzymatic metabolic pathway of drug molecules in humans (Skaanild and Friis, 1997; Soucek *et al*., 2001).

The Large White pig model was therefore selected for the *in vivo* analysis of the Dual-Biotic System due to the its size, physiological and anatomical traits, gastrointestinal flora population and the allowance to withdraw adequate amounts of blood from the cardiovascular system without inducing harmful physiological effects. Current *in vivo* studies for the analysis of probiotic formulations involve utilizing fecal matter for quantification through inoculation onto microbiological agar. While this is a commonly accepted methodology for probiotic formulation efficacy, the possibility of contamination by other

bacteria in the colon can inflate bacterial cell counts leading to inaccurate results (Marteau and Shanahan, 2003). Examples of possible intestinal flora that can provide inaccurate results are *Lactobacillus* and *Bifidobacterium* species of bacteria. These bacteria species are widely used in probiotic research and are naturally found in the colon of humans and pigs and can easily contribute to fecal cell counts (Calinescu and Mateescu, 2008).

Intestinal cannulas have in the past been utilized in nutritional research for the aspiration of intestinal media (Wubben *et al*., 2001). The cannulas are designed specifically to allow for intestinal fluid aspiration directly from the intestinal lumen with the use of modified syringes or needles (Bjornhag and Jonsson, 1984). Intestinal cannulas can therefore also be utilized in probiotic studies where aspiration of intestinal fluid directly from the delivery site would prevent the possible contamination by resident colonic flora. This study therefore utilizes a designed intestinal cannula, manufactured from robust perspex and hard plastic, modified for aspiration of intestinal fluid from a Large White pig. Use of a modified 5mL syringe would allow for the effective aspiration of intestinal fluid without injury to the intestinal lumen of the pig. Inoculation of the aspirated intestinal fluid onto the formulated modified MRS agar would therefore provide an accurate probiotic release profile with the high adhesion potential of the probiotic bacteria ensuring intestinal wall binding and therefore functional health benefits to the host. The required controls are included in the study design to cater for bacterial cell counts due to resident intestinal flora.

7.1.1 Pharmacokinetic modeling and the significance of an *in vitro/in vivo* correlation

Pharmacokinetic modeling provides vital information on the distribution of drug molecules in the human body and can be utilized as an accurate predictor of elimination and therapeutic effectiveness. Two broad categories of pharmacokinetic models currently exist, the non-compartmental and compartmental models. Non-compartmental modeling is based on the premise of a single site of action where little or no distribution of drug takes place. The compartmental model is more complex integrating various different sites of the body, each with its respective coefficients and compartmental volumes. Oral delivery systems primarily conform to the compartmental model, as depicted in Figure 7.1, where the absorption, central and peripheral compartments are independent of each other. Drug release into the central compartment is therefore dependent on absorption from the absorption compartment, most often the intestinal system. The central compartment is primarily responsible for the elimination of drug molecules through hepatic and renal excretion. Intravenous systems, however, are delivered directly to the central compartment where 100% bioavailability occurs with a rapid onset of action. Whilst IV formulations are most effective, the invasive nature of

the delivery system combined with common need for controlled IV delivery results in the utilization of oral delivery systems by a majority of patients.

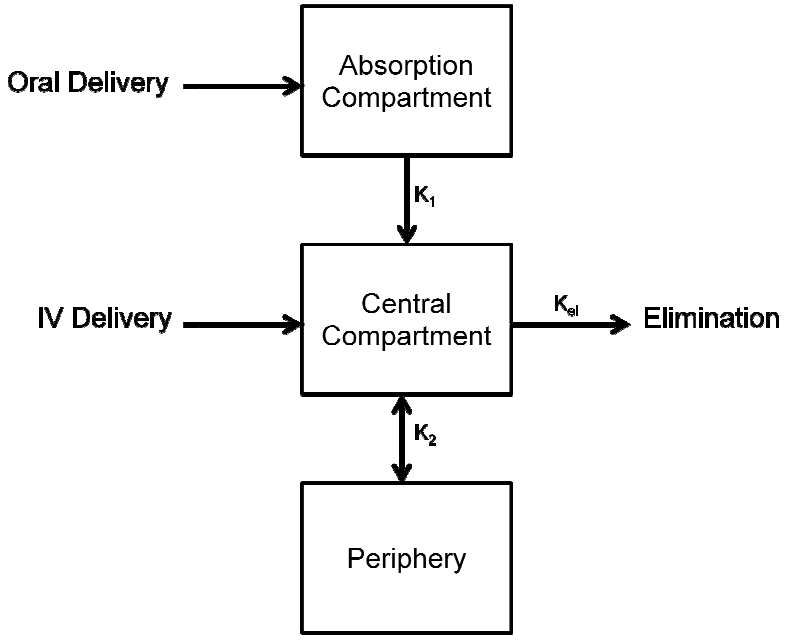


Figure 7.1: Schematic representation of the compartmental distribution of drugs in the human body (adapted from Pourtaheri and Truskey, 2004).

The establishment of a suitable *in vitro* to *in vivo* correlation is also vital for the development of a novel delivery system. This correlation can be utilized for the advancement and modification of a system, without the requirement to conduct thorough *in vivo* studies at each developmental stage. Currently, five correlation levels have been defined by the Food and Drug Administration (FDA) for *in vitro-in vivo* correlations (IVIVC). The premise behind the individual correlations is its ability to accurately predict drug plasma levels at a respective time interval following administration of the delivery system (Emami, 2006; Ostrowski and Baczek, 2010). The five correlation levels as stated by the FDA include:

- Level A is the highest category of correlation and represents a directly associated linear relationship between rate of absorption *in vivo* and dissolution rate *in vitro* at all available time points. The percent of drug absorbed in this correlation may thereafter be calculated by means of model dependent techniques such as the Wagner-Nelson procedure, the Loo-Riegelman method or by model-independent numerical deconvolution. In some cases however, a non-linear relationship may also be appropriate. The advantage of this type of correlation is the ability to utilize a dissolution rate of a drug to accurately predict *in vivo* performance (Emami, 2006).

- Level B is based on the principles of statistical moment analysis, where mean residence time *in vivo* is compared to mean dissolution time *in vitro*. It utilizes all available *in vitro* and *in vivo* data, but it is not considered a direct relationship since any number of *in vivo* curves can produce similar residence time values.
- Single point level C represents a single point relationship between one dissolution parameter (e.g. MDT_{50}) and one pharmacokinetic parameter (e.g. AUC, T_{max} or C_{max}). It is considered to be the weakest category of correlation due to its limited ability to accurately predict *in vivo* release.
- Multiple point level C indicate the existence of a relationship between one or more pharmacokinetic and dissolution parameters at several *in vitro* time points and should be represented by, at minimum, three time points covering the early, middle and late stages of a dissolution profile.
- Level D is a semi-quantitative rank order correlation and is not useful for regulatory purposes. It is not a formal correlation and is generally utilized in the development of formulation or processing procedures (Emami, 2006).

A Level A IVIVC is therefore considered ideal in the development of novel delivery systems and provides an accurate predictability of *in vivo* performance based on *in vitro* release behavior. A Level A IVIVC is evaluated by the predicted error (PE) criteria as defined in FDA guidelines. A reliable Level A correlation exists when an average absolute PE for AUC and C_{max} is below 10% and the PE for the individual formulation does not exceed 15%. The correlation coefficient (R) may also be useful in addition to PE when evaluating a Level A correlation (% *in vivo* absorbed vs. % *in vitro* dissolved). Literature indicates that an R^2 value greater than 0.9 indicates an existing IVIVC (Ostrowski and Baczek, 2010).

This chapter will therefore analyze the formulated Dual-Biotic System in a Large White pig model with intestinal fluid aspirated through a surgically implanted intestinal cannula, in conjunction with blood samples withdrawn through an intra-jugular catheter, at various time intervals. In addition, compartmental and non-compartmental analyses were employed as well as a Level A IVIVC correlation analysis to ascertain the pharmacokinetic properties and clinical capabilities of the Dual-Biotic System.

7.2 Materials and Methods

7.2.1 Materials

Lactobacillus acidophilus ATCC 314 was purchased from Davies Diagnostics (Randburg, Gauteng, South Africa). Raw egg ovalbumin was obtained from commercial chicken eggs (Nulaid®, Grade 1, Grain Fed) purchased from Pick n Pay® Stores (Johannesburg, Gauteng, South Africa). Genipin, kollicoat MAE100P, amoxicillin trihydrate and nystatin were purchased from Sigma-Aldrich (St Louis, MO, USA). Glyceryl monostereate was purchased from Holpro Analytics (Johannesburg, Gauteng, SA). MRS Agar was purchased from Oxoid (Hampshire, UK). All other chemicals were of analytical grade and used as received.

7.2.2 Preparation of the Dual-Biotic System for *in vivo* analysis

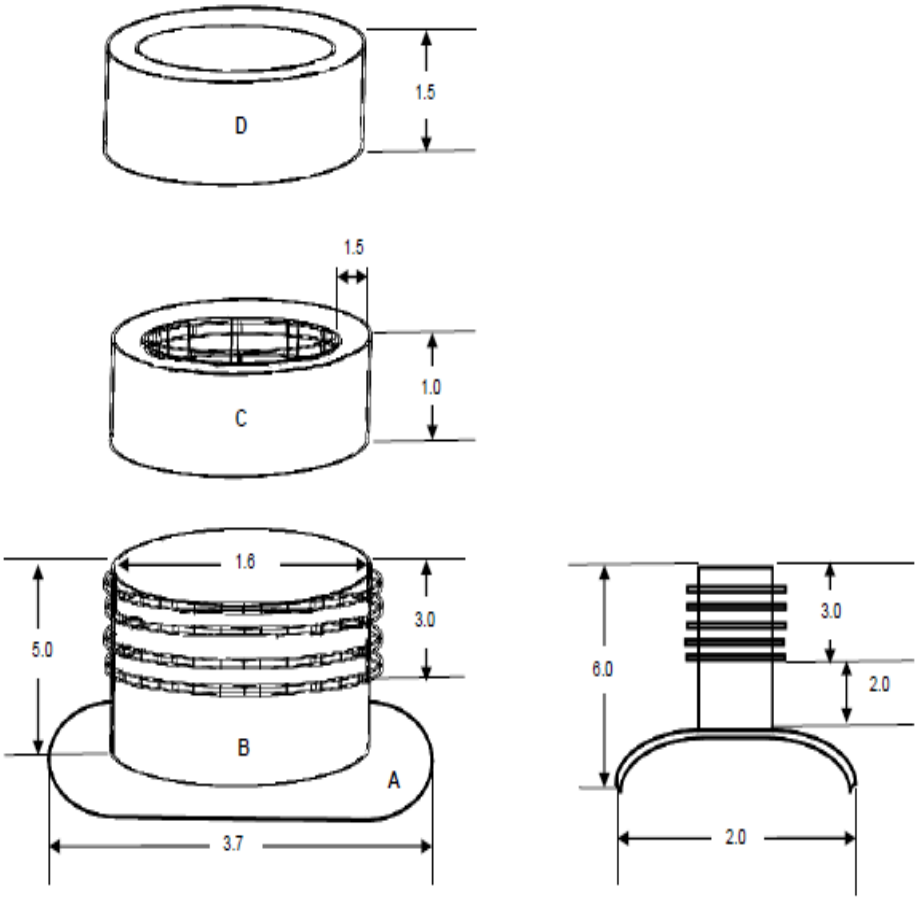
The enteric coated Dual-Biotic System was formulated as stated in Chapter 5, Section 5.2.15. Lactose granules (150mg) compressed at a force of 3 tons were utilized as a filler to maintain capsule integrity and shape during oral administration via the intra-gastric tube.

7.2.3 Quantification of the viable probiotic bacteria contained within the conventional probiotic formulation

The number of viable bacteria contained in the commercially available *Lactobacillus* probiotic product was determined through serial dilution as described in Chapter 3, Section 3.2.2.

7.2.4 Design and manufacture of a stable intestinal cannula for the aspiration of intestinal fluid from the intestinal ileum of a Large White Pig

The intestinal cannula was manufactured utilizing a hard plastic cap, collar and body placed over a perspex flange molded to fit against the intestinal wall of the Large White pig (Figure 7.2a). The inside surface of the cap contained a robust rubber seal to prevent leakage from the cannula, in addition to preventing damage of the cannula cap by intestinal contents. The width of the designed cannula cap and collar were kept to a minimum to prevent forced removal by the pigs after surgical implantation. Sufficient thread was, however, allowed for a secured fit onto the cannula body. Prior to surgical implantation, all intestinal cannulas were radiation sterilized to ensure sterility (Sterilization certificate can be found in Appendix E). Evaluation of the intestinal cannula after gamma sterilization (Figure 7.2b) revealed no distinct changes in cannula integrity or structure.



(a)

(b)

Figure 7.2: Schematic representation of the manufactured intestinal cannula for intestinal fluid aspiration (a; all dimensions are in cm) and a digital image of the sterilized intestinal cannula (b).

7.2.5 Surgical implantation of an intra-jugular catheter and intestinal cannula

Prior to the surgical implantation of the intra-jugular catheter and intestinal cannula, each Large White pig was anaesthetized (Figure 7.3a) using ketamine HCl (11mg/kg) and midazolam (0.3mg/kg) and maintained (Figure 7.3b) under medical oxygen (12%) and isoflurane gas (2%) for the duration of the procedure (Hodgson, 2007; Linkenhoker *et al*., 2010). Each pig was weighed prior to the anesthesia to ascertain the dosage requirements of the respective pig. The pigs were thereafter shaved on the surgical sites prior to commencement of the surgical procedure (Figure 7.2c) with a Jelco® peripheral catheter inserted into a vein of the right ear for rapid delivery of drugs during surgery (Figure 7.2d). All surgical procedures were undertaken by Professor K Erlwanger of the School of Physiology, University of the Witwatersrand with assistance by the surgical staff of the Central Animal Services, University of the Witwatersrand.

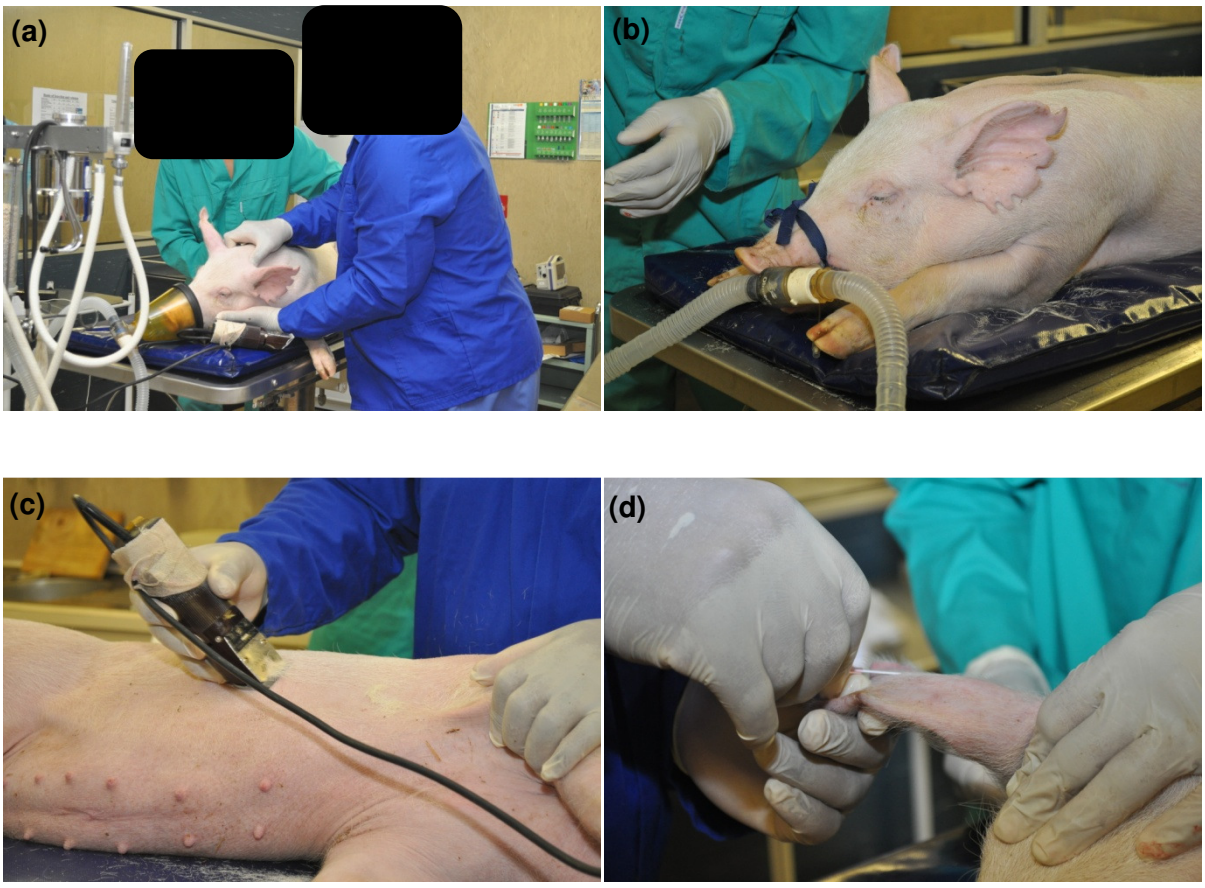


Figure 7.3: Digital images of the surgical preparation procedure.

The intestinal cannula was surgically implanted under aseptic conditions into the intestinal ileum of the Large White pig by making a 6cm incision 15cm from the ileo-ceacal junction (as

depicted in Figure 7.4) and extruding the cannula body through the exterior wall of the pig. For the surgical implantation, an initial incision was made on the flank of the pig for exposure of the intestinal ileum (Figure 7.5a-b). The blood supply to the implantation area was thereafter isolated (Figure 7.5c) prior to the incision and implantation of the cannula within the intestinal lumen (Figure 7.5d). The intestinal wall was then sewn over the flange of the cannula. An extrusion point was then made with the cannula cap and collar removed through the extrusion site (Figure 7.5e-f). The initial incision site and extrusion area was thereafter sutured to promote rapid healing of the surgical area (Figure 7.5g).

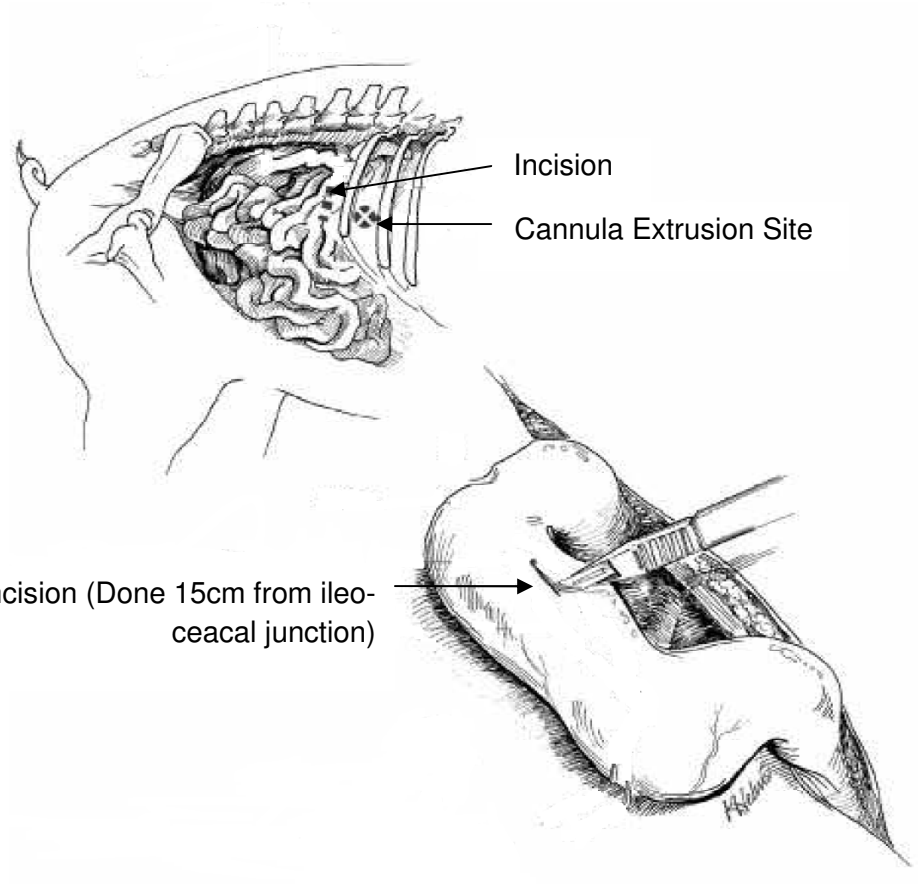


Figure 7.4: A diagrammatic representation of the surgical implantation of the intestinal cannula in the ileum of the Large White pig (Adapted from Wubben *et al*. 2001).

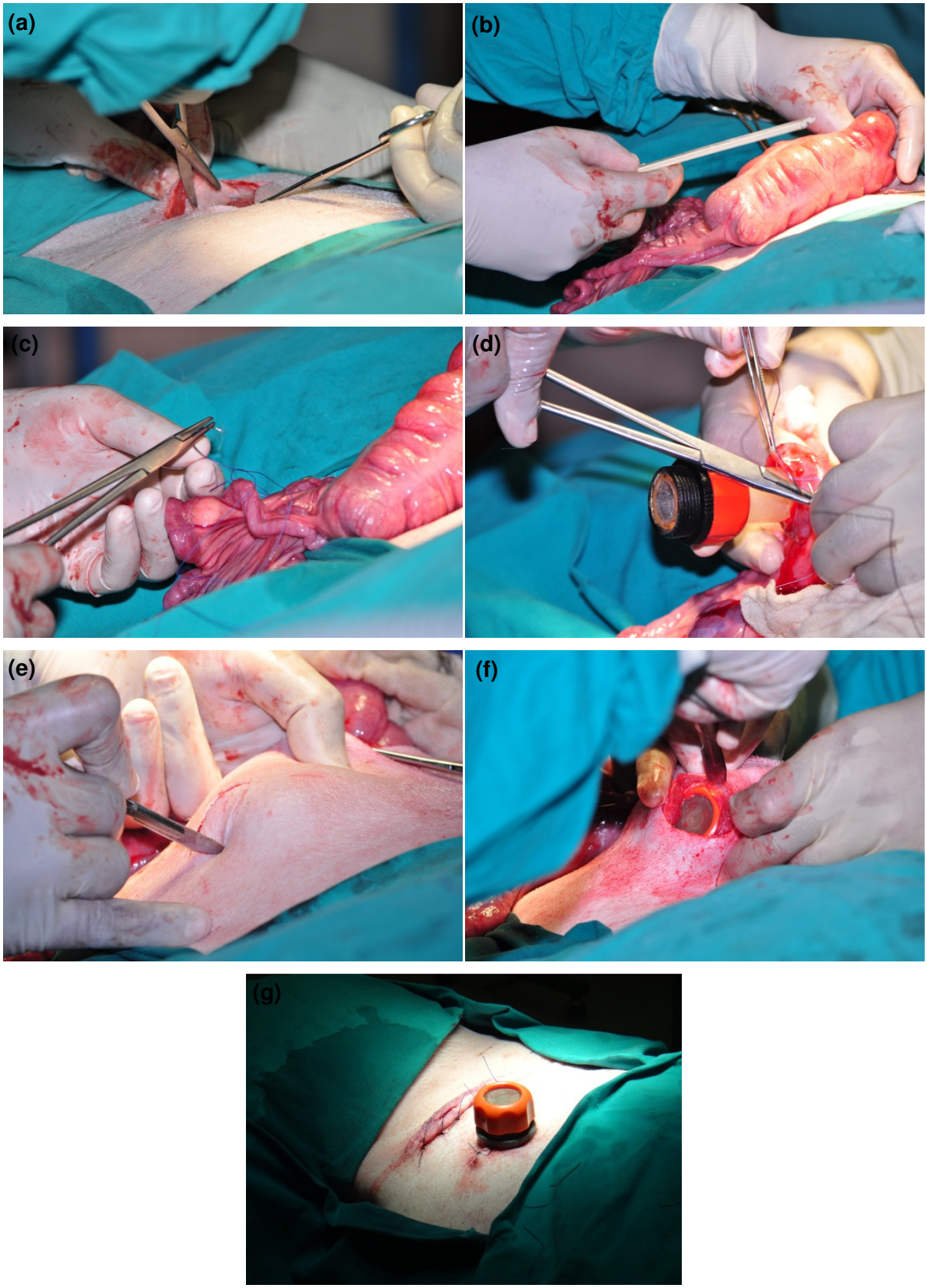


Figure 7.5: Digital images of the surgical implantation of the intestinal cannula.

The catheter implantation procedure was undertaken using a 7 gauge double lumen 60cm catheter (CS-28702, Arrow Deutschland GmdH, Erding, Germany) inserted into the left jugular vein. The jugular vein was exposed by an incision made dorsal to the jugular groove on the left lateral aspect of the neck (Figure 7.6a). The jugular vein was thereafter isolated by blunt dissection with 10cm of the catheter inserted into the lumen of the vein (Figure 7.6b-c). The remaining 50cm of catheter was then tunneled subcutaneously to the exit point cranial to the dorsal aspect of the scapula using a trocar with the catheter ports sutured onto the exterior surface of the skin (Figure 7.6d). Heparin saline (5000 iu/L) was then flushed into the catheters to remove residual blood as well as to maintain catheter integrity. All surgical sites were then sutured to promote rapid healing (Figure 7.6e-f) with flushing of the catheter conducted twice daily until completion of the study.

Prior to *in vivo* analysis, all pigs were given post-operative prophylactic treatment with benzathine penicillin and analgesic treatment with buprenorphine (0.05mg/kg I.M.) and carpofen (4mg/kg I.M.). Topical antiseptic ointment was applied twice daily to the surgical sites for the prevention of superficial infections. Sufficient time was given for healing of surgical sites as well as for washout of the respective post-operative treatment prior to conduction of the study. All pigs were fitted with an elastic vest, with the catheter ports and intestinal cannula covered, to prevent forced removal by the pigs during the course of the study (Figure 7.7). All pigs were placed in isolated pens with smooth walls for the prevention of forced cannula removal with food and water available *ad libitum*. The entire post operative course was documented using set protocols where fluctuations in body weight, eating patterns and movement were reported and accordingly addressed.

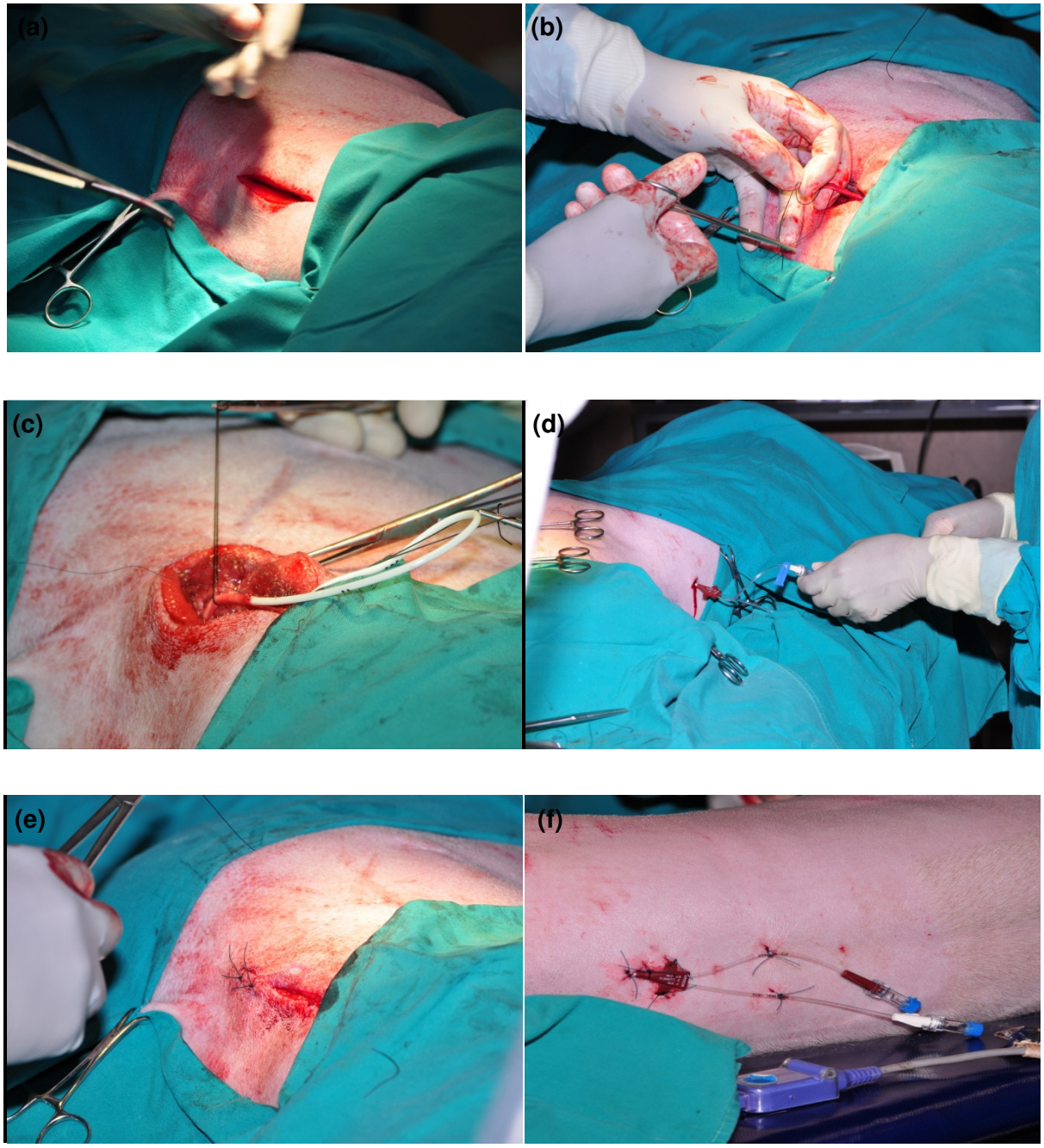


Figure 7.6: Digital images of the surgical implantation of the intra-jugular catheter.

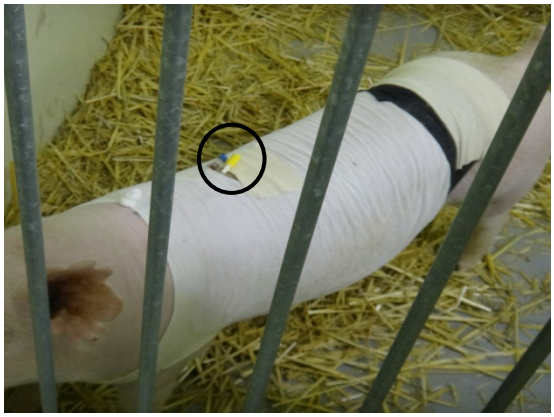


Figure 7.7: Digital image of the elastic vest utilized with the intra-jugular catheter ports depicted.

7.2.6 *In vivo* analyses utilizing a Large White pig model

In vivo analysis was conducted on fourteen female Large White pigs (approx. 30kg), each implanted with the intestinal cannula and intra-jugular catheter as stated in Section 7.2.5, evenly allocated into two groups (Figure 7.8). Within each group, five pigs served as the experimental component with the remaining two pigs serving as controls. Group 1 was designated as the experimental group that was administered with the Dual-Biotic System at time 0 with Group 2 serving as the conventional group which was administered initially with a conventional *Lactobacillus* product, and thereafter with a conventional antibiotic formulation concomitantly with the conventional probiotic product. Each analysis was conducted over a 24 hour period with each group run sequentially to allow for the rapid utilization of the pig models. After completion of the study, each pig was euthanized with an overdose of sodium pentobarbitone (>200mg/kg) administered via the intra-jugular catheter.

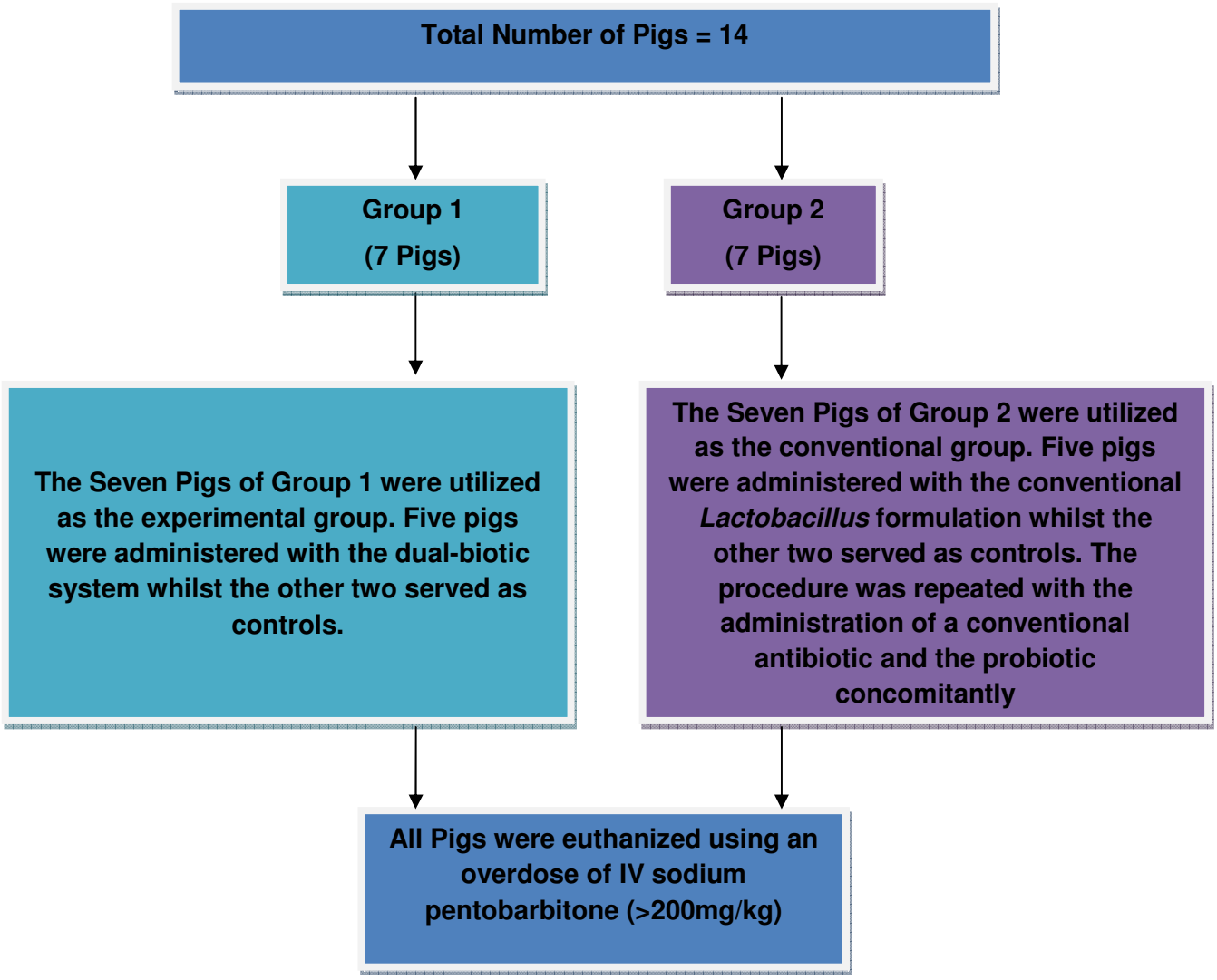


Figure 7.8: A flow diagram depicting the allocation of pigs into the experimental and conventional groups.

7.2.6.1 *In vivo analysis of the dual-biotic and conventional delivery systems*

In vivo release analysis was conducted in three sets. Set 1 consisted of Group 1 (n=5) administered with Dual-Biotic System, Set 2 consisted of Group 2 (n=5) administered with a conventional antibiotic product containing amoxicillin trihydrate equivalent to 250mg amoxicillin base and a conventional *Lactobacillus* probiotic product concomitantly. Set 3 consisted of Group 2 administered with the conventional *Lactobacillus* probiotic product only. In each set, 2 pigs served as controls. The controls for set 1 included the administration of 250mg amoxicillin trihydrate incorporated into the Dual-Biotic System without the addition of the delayed-release system. Controls for set 2 included the administration of the conventional antibiotic formulation only with the controls for set 3 including placebo formulations containing lactose. For the administration of the respective oral delivery systems, an intra-gastric tube was utilized. The procedure for oral administration included

anesthetizing the dosed pigs with ketamine HCl (11mg/kg) and midazolam (0.3mg/kg) with the pigs maintained under medical oxygen (12%) and isoflurane gas (2%) as depicted in Figure 7.9a. Xylazine was thereafter sprayed into the esophagus of the pig with the intra-gastric tube coated with KY jelly for lubrication, inserted into the stomach (Figure 7.9b-c). The pig was thereafter raised with the respective formulation(s) inserted into the tube and administered into the stomach with the aid of 15mL pour water.

Figure 7.9: Digital images of the procedure utilized for the oral administration of the dual-biotic and conventional systems.

7.2.6.2 *Blood and intestinal fluid sampling*

Blood and intestinal fluid sampling was undertaken at time intervals 0, 1, 2, 3, 4, 6, 8, 10, 12, 18 and 24 hours respectively. Blood samples were aspirated via the intra-jugular catheter by flushing the catheter with 5mL heparin saline, withdrawing and discarding the heparin saline and thereafter aspirating 4mL of blood. Heparin saline (5mL) was thereafter injected into the port to maintain catheter integrity. All blood samples were placed in heparinised vacutainers (Improvacuter®, Guangzhou Improve Medical Instruments, Guangzhou, China) prior to plasma extraction. For the extraction of plasma, the blood samples were centrifuged at

3000rpm for 15 minutes, the plasma supernatant extracted and stored at -80℃. Intestinal fluid samples (2mL) were aspirated via the intestinal cannula utilizing a sterile syringe with the tips removed. This was conducted to minimize damage to the intestinal wall during sample aspiration. After aspiration of the intestinal fluid, all samples were placed in sterile tubes and stored at 4℃ in a refrigerator until analysis.

7.2.7 *In vivo* amoxicillin quantification utilizing Ultra-Performance Liquid Chromatography

UPLC analysis of the aspirated plasma samples were undertaken utilizing a Waters Acquity® UPLC system (Waters®, Milford, MA, USA) containing an Acquity UPLC BEH C18 column (50 mm×2.1mm, i.d., 1.7µm particle size, Waters) set to a temperature of 40℃, coupled with a photodiode array detector (PDA) and Empower® Pro software (Waters®, Milford, MA, USA). Chromatographic conditions employed were based on the method outlined by Carlier and co-workers (2012) with modifications. The mobile phase consisted of 0.1% formic acid in double deionized water and pure acetonitrile at a ratio of 80:20. The analysis was conducted using an injection volume of 10µL at an isocratic flow rate of 0.35mL/min over a run time of 4 minutes. The wavelength for amoxicillin detection was maintained at 273nm. Methyl-paraben at a concentration of 20µg/mL was utilized as the internal standard. The solvents utilized for priming and maintenance of the UPLC prior to and during plasma amoxicillin quantification are listed in Table 7.1 with a typical UPLC profile and 3-D plot for plasma amoxicillin quantification depicted in Figure 7.10a and 10b respectively.

Table 7.1: Solvents employed during UPLC analysis.

Solvent Line	Solvent Concentrations
Strong wash	Acetonitrile (ACN) (90%) and double deionized water (10%)
Weak wash	Acetonitrile (10%) and double deionized water (90%)
Solvent A line	Formic acid (0.1%) in double deionized water
Solvent B_1 line	Acetonitrile (100%)
Solvent B_2 line	Methanol (100%)

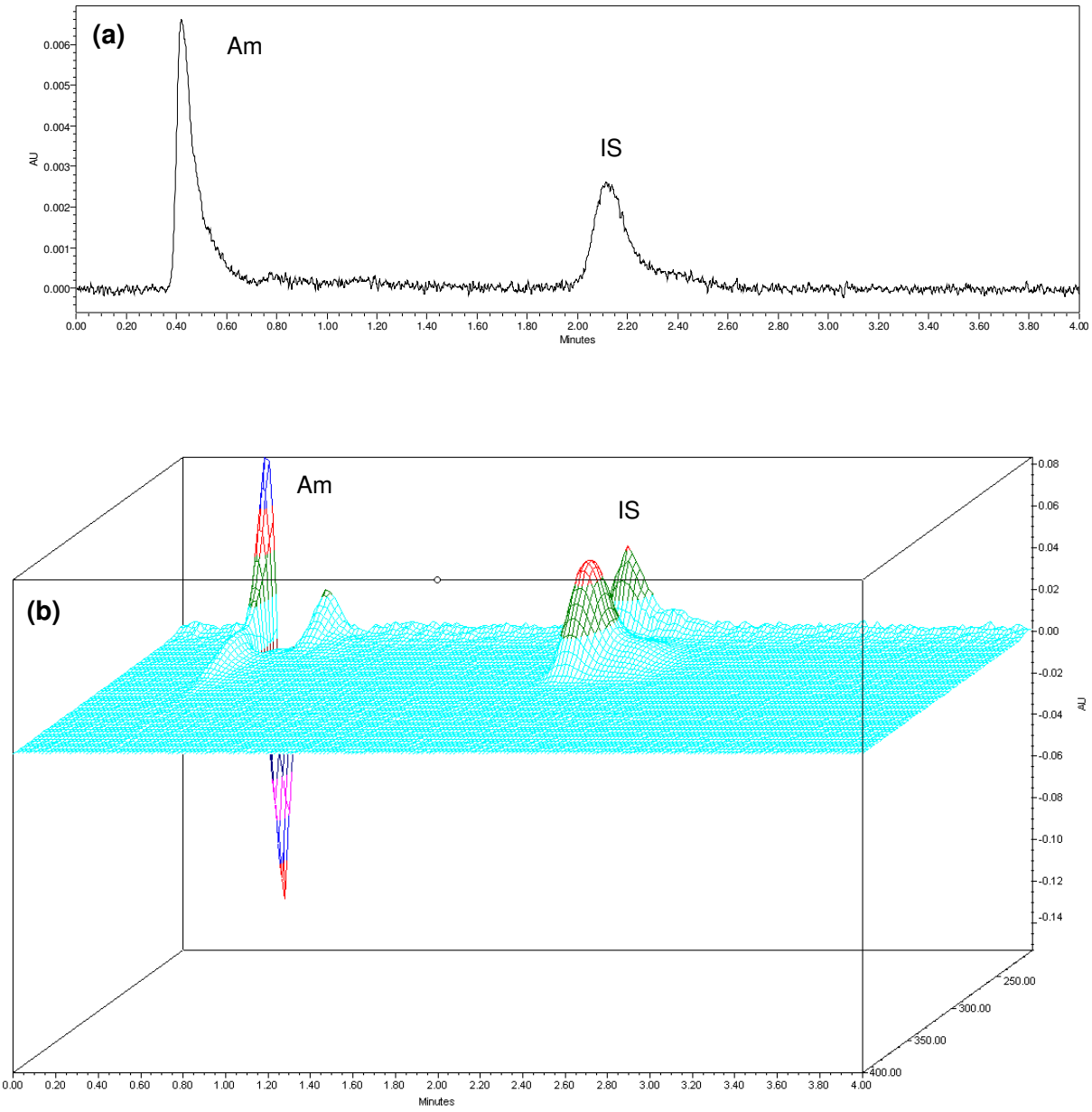


Figure 7.10: A typical UPLC profile conducted on (a) *in vivo* plasma samples and (b) a UPLC-derived 3-D plot. (Am= Amoxicillin and IS= Internal Standard).

7.2.8 Sample preparation utilizing Liquid-Phase extraction

All plasma samples were subjected to a simple liquid-phase extraction procedure prior to UPLC analysis. The liquid-phase extraction procedure was undertaken by adding 10µg of methyl-paraben to 100µL of plasma sample and vortexing for 15 seconds (Vortex-Genie 2; Scientific Industries Inc., Bohemia, New York). The plasma solution was thereafter transferred into a sterile 1.5mL eppendorf prior to the addition of pure acetonitrile (500µL). The solution was vortexed for 1 minute and then centrifuged at 10000x*g* for 10 minutes (TG16-WS, Nison Instrument Ltd., Shanghai, China). The supernatant containing amoxicillin was thereafter removed. The procedure was repeated for the complete removal of plasma

proteins. The supernatant was thereafter passed through a 0.22µm Millipore® filter into UPLC injection vials.

7.2.8.1 *Validation of the extraction procedure*

The liquid-phase extraction procedure was validated by calculating the percentage yield of amoxicillin as stated in Equation 7.1. This was undertaken by spiking 0.01µg of amoxicillin into 100µL of blank plasma, vortexing the solution for 15 seconds and extracting the amoxicillin utilizing the liquid-phase extraction procedure stated in Section 7.2.8. This was compared to a solution of 0.01µg in 100µL mobile phase and the percentage yield thus calculated. Inter-day (n=3) and intra-day variability (n=3) of the extraction procedure was also determined.

$$\%\, yield = \frac{Amoxicillin_{blood}}{Amoxicillin_{mobile}}\, x\, 100 \qquad \text{Equation 7.1}$$

7.2.8.2 *Preparation of calibration standards and construction of an Ultra-Performance Liquid Chromatography amoxicillin calibration curve*

Amoxicillin standards ranging from 0.02-0.1µg/mL were prepared by spiking the respective concentration of amoxicillin into 100µL of blank plasma and vortexing the mixture for 15 seconds. All plasma standards utilized were thereafter stored at -70℃ and subsequently thawed to room temperature (21.05±0.5℃) to coincide with the *in vivo* sample storage conditions utilized. Sample extraction and UPLC analysis was thereafter performed as stated in Section 7.2.8 with a calibration curve formed.

7.2.9 Cell viability analysis on the aspirated intestinal fluid samples

Cell viability analysis was conducted on all aspirated intestinal fluid samples to ascertain the initial decrease in intestinal flora counts due to the antibiotic bactericidal effects with a subsequent increase due to supplementation by the Dual-Biotic System. The analysis was conducted utilizing two quantification protocols, the luminescence protocol which would depict bacterial ATP increases and inoculation onto the modified MRS agar which would ascertain if the microbial ATP increases determined were due to an increase in *Lactobacilli*. The luminescence quantification was performed by a 1:100 dilution of intestinal fluid samples in sterile water with the luminescence thereafter recorded as previously stated in Chapter 3, Section 3.2.2.2.

The increases in *Lactobacilli* counts were confirmed through a 1:100 dilution of intestinal fluid samples in sterile water and inoculation onto the *Lactobacillus*-specific agar as prepared in Chapter 6, Section 6.2.2.3. The inoculated agar plates were incubated at 37℃ for 48 hours under anaerobic candle jar conditions with visual quantification of the CFUs thereafter performed.

7.2.10 Pharmacokinetic modeling of *in vivo* amoxicillin data utilizing non-compartmental and compartmental algorithms

Pharmacokinetic modeling was achieved using PKSolver, a menu-driven add-in program for Microsoft Excel, to undertake non-compartmental and compartmental analysis of the *in vivo* plasma amoxicillin data (Zhang *et al.*, 2010).

Non-compartmental analysis is extensively utilized in pharmacokinetic analysis. Calculation of the area under the zero and first moment curves from 0 to last time t ($AUC_{0\text{-}t}$, $AUMC_{0\text{-}t}$) is generally undertaken using the linear trapezoidal, log-linear trapezoidal or linear up/log down methods. In addition, the terminal elimination slope, z, can be calculated utilizing either user-defined terminal data points or automatically estimated through regression analysis with the largest adjusted R^2, as per Equation 7.2, determined. Area under the zero and first moment curves from the last sampling time to infinity (AUCt-∞, AUMCt-∞) were calculated based on the last observed or predicted concentration employing Equations 7.3 and 7.4 respectively:

$$Adjusted\ R^2 = \frac{(1-R^2)(n-1)}{(n-2)} \qquad \text{Equation 7.2}$$

Where:
n is the number of data points in the regression and
R^2 is the square of the correlation coefficient.

$$AUC_{t-\infty} = \frac{C_{last}}{\lambda_z} \qquad \text{Equation 7.3}$$

Where:
C_{last} is the last observed or predicted concentration and
λ_z is the terminal elimination slope.

$$AUMC_{t-\infty} = \frac{C_{last}\, x\, t_{last}}{\lambda_z} + \frac{C_{last}}{\lambda_z^2} \qquad \text{Equation 7.4}$$

Where:

t_{last} is the time of the last observed or predicted concentration.

Additional parameters that are calculated through non-compartmental analysis include Elimination half-life ($t_{1/2}$), Peak amoxicillin time (T_{max}), Peak amoxicillin concentration (C_{max}), mean residence time (MRT) and clearance (Cl).

The complex absorptive and distribution processes involving the oral administration of therapeutic actives necessitates the selection of a suitable pharmacokinetic model for the fitting of concentration-time data. The selection of an appropriate model, however, requires the use of specific diagnostics such as the correlation coefficient, sum of squares of residuals (SS), standard error of weighted residuals (SE), Akaike's Information Criterion (AIC) and Schwarz's Bayesian Criterion (SBC). AIC and SBC, however, hold the greatest significance in determining the most suitable model and can be calculated utilizing Equations 5.7 and 7.5 respectively (Zhang *et al*., 2010).

$$SBC = -2L_R + Slog(N - R) \qquad \text{Equation 7.5}$$

Where:

L_R is the restricted log-likelihood function evaluated at final fixed parameter estimates and the final variance parameter estimates,

N is the number of observations used,

r is the rank of the fixed effects design matrix X and

s is the rank of the fixed effects design matrix X plus the number of parameters in θ.

Compartmental analysis was further utilized to quantitatively evaluate and predict the *in vivo* fate of amoxicillin once absorption was completed by modeling the concentration-time data with a suitable pharmacokinetic compartment algorithm. Administration of the Dual-Biotic System was considered as a single extravascular dose with the site-specific nature of the delivery system resulting in an absorption lag. For this reason, four pharmacokinetic algorithms were utilized and include:

- Non-compartmental model
- One compartment model
- One compartment model with T_{lag}
- Two compartment model with T_{lag}

In addition to the pharmacokinetic modeling of amoxicillin plasma data, *L. acidophilus* release data were also modeled. Whilst the *L. acidophilus* was not absorbed into the cardiovascular system, pharmacokinetic properties such as AUC, MRT and the elimination constant would provide useful information into the release of the probiotic bacteria and the rate of probiotic decrease in intestinal fluid. The Dual-Biotic System was considered as a single extravascular dose with a release lag. Modeling of bacterial release in intestinal fluid has been previously performed using multi-compartmental models and was therefore undertaken utilizing the four pharmacokinetic algorithms stated for the modeling of *in vivo* amoxicillin release (Marteau and Shanahan, 2003).

7.2.11 Establishment of an *in vitro-in vivo* correlation

WinNonLin® software (V5.3 with IVIVC Toolkit Build 20091211139, Pharsight Software, Statistical Consultants Inc., Apex, NC, USA) was employed for establishment of a Level A IVIVC. A Level A IVIVC, as previously stated, is a correlation between the *in vitro* drug dissolution profile and *in vivo* drug absorption profile. Input data for this correlation therefore included *in vitro* amoxicillin and *L. acidophilus* release data, in addition to the relevant pharmacokinetic data. For the evaluation of an IVIVC model, development and validation is required. The development of level A IVIVC model therefore follows a two-stage process:

1. Deconvolution: the observed fraction of the drug absorbed is estimated based on the Wagner-Nelson method. The IVIVC model is thereafter developed using the observed fraction of the drug absorbed and that of the drug dissolved. Based on the IVIVC model used, the predicted fraction of the drug absorbed is thereafter calculated from the observed fraction of the drug dissolved.
2. Convolution: the predicted fraction of the drug absorbed is then convolved to the predicted concentrations instituting the convolution method. The predictability of a level A correlation thereafter focuses on estimating the percent prediction error (%PE) between the observed and predicted concentration profiles, such as the difference in pharmacokinetic parameters (C_{max} and $AUC_{0-\infty}$).

7.3 Results and Discussion

7.3.1 Viable bacterial counts of the conventional probiotic formulation

Evaluation of the conventional probiotic formulation determined that approximately 242mg probiotic powder contained 2.30×10^9 viable *Lactobacillus* cells. This, as with the Dual-Biotic System, maintained the bacterial cell counts required for therapeutic requirements. The result, however, revealed a significantly higher bacterial cell count than what was included in the dual-biotic formulation. This was significant as it would determine if the number of viable bacteria contained within the formulation affected release profiles and bacterial survival.

7.3.2 Validation of the extraction procedure

Evaluation of the percentage yield of the extraction process determined a 94.32% amoxicillin plasma concentration when compared to the spiked mobile phase. This result therefore determined the effectiveness of the utilized liquid-phase extraction procedure undertaken, with a high ratio of plasma amoxicillin injected during UPLC quantification. Inter-day and intra-day variability analysis determined minimal differences between sample sets (2.81% and 2.53% variation respectively). The reproducibility of the extraction process was thus determined, validating the use of the liquid-phase extraction procedure for *in vivo* plasma sample preparation.

7.3.3 A calibration curve for the quantification of plasma amoxicillin concentrations

The constructed calibration curve determined a gradient value of 34.8471 at 273nm (Figure 7.11). The use of this calibration curve was validated due to its excellent linearity with a R^2 value of 0.99 determined. The constructed calibration curve therefore utilized in all calculations for the determination of plasma amoxicillin concentrations.

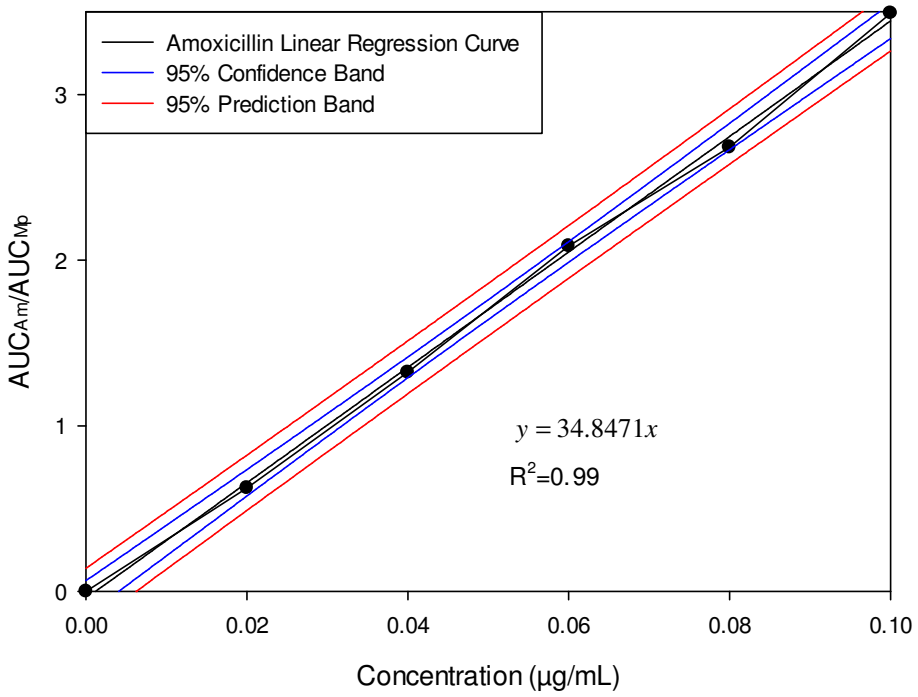


Figure 7.11: UPLC calibration curve of known plasma amoxicillin concentrations.

7.3.4 *In vivo* profiles of the Dual-Biotic System

In vivo analysis of the Dual-Biotic System determined the effectiveness of the dosage form to protect concurrently administered *L. acidophilus* probiotic bacteria against the bactericidal effects of amoxicillin. An initial lag in amoxicillin absorption was noted which can be attributed to the gastric-emptying required for the site-specific delivery system to enter the small intestine. The subsequent release of amoxicillin resulted in a rapid absorption of amoxicillin (Figure 7.12) to a maximum concentration (C_{max}) at approximately 2.65 hours (T_{max}). This correlates with previous studies that have determined rapid absorption of amoxicillin (Witkowski *et al.*, 1982). The minimal lag seen can be further correlated with *ex vivo* release studies conducted in Chapter 6, Section 6.3.9, where the polymer coating employed dissolved at a faster rate than during *in vitro* release studies. The plasma concentration of amoxicillin between Hours 0 and 8 prior to the next dose of antibiotic is also of significance. The effectiveness of an antibiotic *in vivo*, especially with time-dependent antibiotics such as amoxicillin, is reliant on its ability to maintain minimum inhibitory concentrations throughout the treatment period. The plasma amoxicillin concentration at Hour 8 of approximately 2µg/mL has been documented to be effective against *E.coli* (MIC= 2µg/mL), *Enterococcus faecalis* (MIC= 0.5µg/mL), *Haemophilus influenza* (MIC= 2µg/mL), *S. aureus* (MIC= 0.5µg/mL) and *Streptococcus pneumonia* (MIC= 0.06µg/mL), commonly treated bacterial infections utilizing amoxicillin (RxList, 2011). The rapid elimination of

amoxicillin, to a concentration undetectable by UPLC at 18 hours, was also documented with amoxicillin having a short plasma half-life of up to 2 hours (He *et al*., 2010).

Upon viability analysis utilizing luminescence, the effectiveness of the delayed release system was also determined. An initial decrease in bacterial cell counts was determined at Hour 4 as a result of bacterial destruction by amoxicillin. Amoxicillin was fully absorbed by Hour 3, as determined in the amoxicillin profile, however, the delay in detection of bacterial destruction is due to the site of intestinal fluid aspiration further along the intestinal tract. The site of cannulation is, however, validated as it would provide valuable data on probiotic release as opposed to a higher cannulation point where bacterial detection is dependent on a lack of intestinal transit. The subsequent increase in bacterial cell count was thereafter seen, due to the delayed release system supplementing intestinal flora, with maximum bacterial viability seen at Hour 6. Upon transit of the intestinal fluid pass the cannula site, a gradual decrease in bacterial cell counts to equilibrium after 24 hours occurs. This equilibrium is due to intestinal flora balance occurring, which is attributed to the increase in intestinal flora through supplementation, resulting in a higher baseline viability than prior to administration of the Dual-Biotic System. An initial increase in intestinal cell counts (Hour 1) was determined throughout all analyses including the placebo set and in was in correlation with the activity and dietary consumption of the pigs. The control of amoxicillin only detailed a rapid decrease in cell viability in correlation with the Dual-Biotic System with a cell viability after 24 hours lower than the baseline prior to administration.

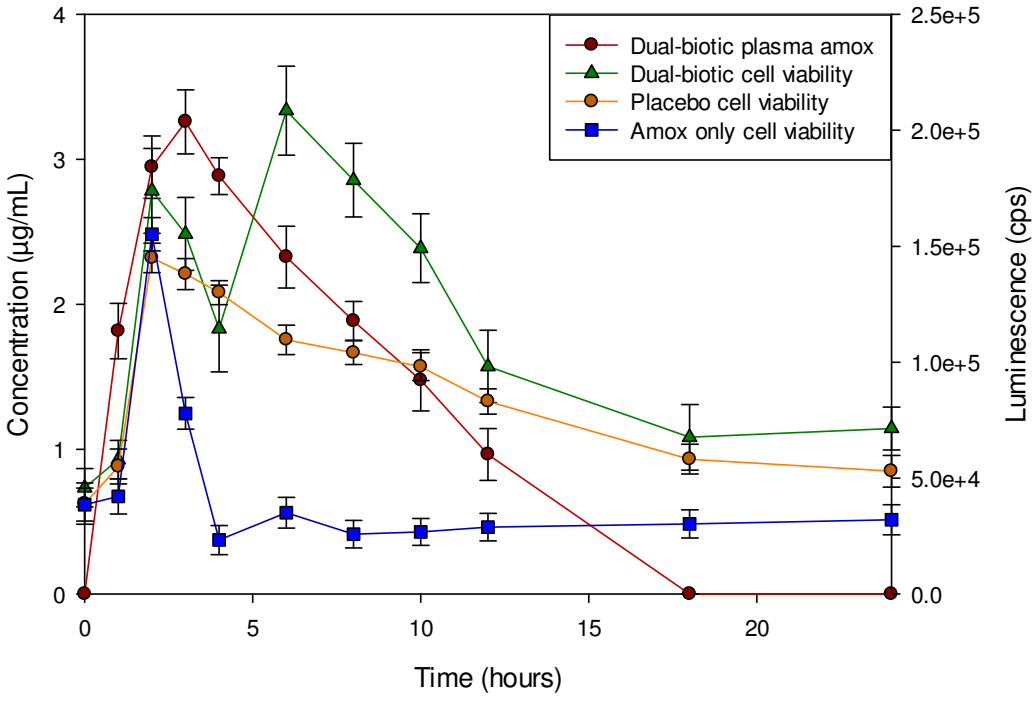


Figure 7.12: *In vivo* release profiles of the Dual-Biotic System (n=5).

7.3.5 *In vivo* profiles of the conventional probiotic and antibiotic formulations

In vivo analysis of the conventional amoxicillin and probiotic formulations revealed results comparable to that seen with the Dual-Biotic System. When administered concurrently with the antibiotic, the probiotic formulation failed to provide a significant increase in bacterial cell counts (Figure 7.13), due to the bactericidal effects of amoxicillin, as well as the bacterial destruction associated with gastric conditions (Albertini *et al*., 2010). *Lactobacillus* bacteria do harbor a natural resistance to gastric conditions, however, this resistance is time dependent with a significant amount of bacterial death occurring upon exposure to gastric conditions (Krasaekoopt *et al*., 2004; Maragkoudakis *et al*., 2006).

The controls of an amoxicillin only administration and a probiotic only administration were added as the comparative analysis. The analysis utilizing the amoxicillin only administration determined a significant decrease in bacterial cell counts (Figure 7.13). This decrease was greater than that seen when the probiotic formulation and antibiotic formulation were administered concomitantly. This result therefore determined that a small proportion of probiotic bacteria do survive exposure to antibiotics if administered concurrently, however, the large decrease in probiotic cell counts prevents the actions associated with probiotic supplementation, warranting the practice of antibiotic and probiotic administration separation. Conventional amoxicillin plasma concentrations revealed further comparisons with the Dual-Biotic System. Due to the lack of site specific delivery, amoxicillin plasma concentrations results revealed rapid release and absorption of the administered antibiotic (Figure 7.13). The rapid absorption revealed a T_{max} of approximately 1.5 hours, which is expected from amoxicillin trihydrate delivery. Due to the rapid absorption, rapid elimination occurs (with no amoxicillin concentration determined by Hour 12) which was in correlation with the Dual-Biotic System analysis. From this analysis, it was therefore determined that the Dual-Biotic System maintained the absorption and elimination characteristics seen in traditional amoxicillin therapy, with the added effect of intestinal flora supplementation when administered concurrently.

The control of probiotic only caused large increases in intestinal cell counts due to the viable bacterial cells delivered to the intestinal tract. The increase, however, was not as large as the Dual-Biotic System, which is due to gastric exposure prior to intestinal transit. Furthermore, due to the initial probiotic load of the conventional probiotic formulation being significantly higher than that of the Dual-Biotic System, this analysis proves the effectiveness of the Dual-Biotic System to maintain probiotic cell viability for delivery to the intestinal tract.

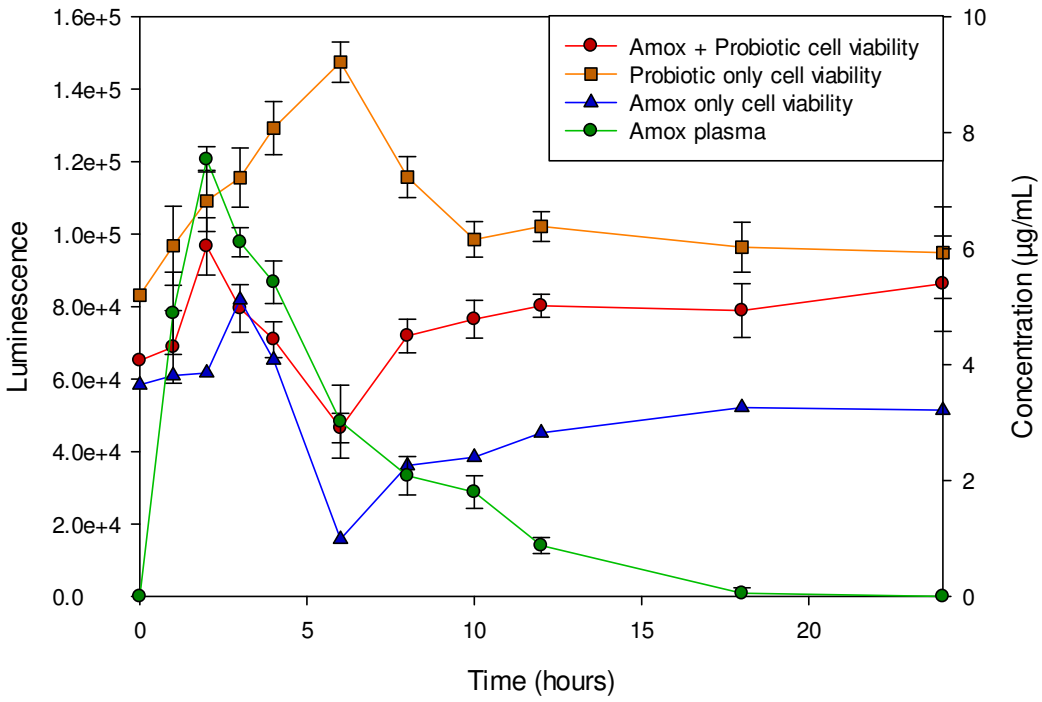


Figure 7.13: *In vivo* release profiles of the analyzed conventional formulations (n=5).

7.3.6 Evaluation of the aspirated *in vivo* intestinal fluid samples utilizing the *Lactobacillus*-specific agar

Evaluation of the aspirated intestinal fluid samples revealed significant increases in *Lactobacillus* bacterial CFUs from the Dual-Biotic System (Figure 7.14). This was in correlation to the results determined in the luminescence quantification, with a significant decrease in *Lactobacillus* cell count, due to amoxicillin's antibacterial properties, prior to an increase in *Lactobacillus* CFUs. Intestinal flora stabilization was also determined with a bacterial baseline improved after administration of the Dual-Biotic System.

Similar correlations were seen during the conventional analysis and in the administered controls with a significant decrease in *Lactobacilli* count determined after concomitant administration of the antibiotic and probiotic products and administration of the antibiotic product only. Evaluation of the probiotic product revealed a distinct increase in *Lactobacilli* CFUs, however, as determined through luminescence quantification, this was significantly less than the dual-biotic-system. The results determined during the luminescence quantification analyses was therefore validated through use of inoculation of intestinal fluid samples onto the *Lactobacillus*-specific agar. This evaluation therefore proved that increases in intestinal fluid bacterial cell counts can be attributed to an increase in *Lactobacilli* concentration, thereby accurately determining the effectiveness of the Dual-Biotic System.

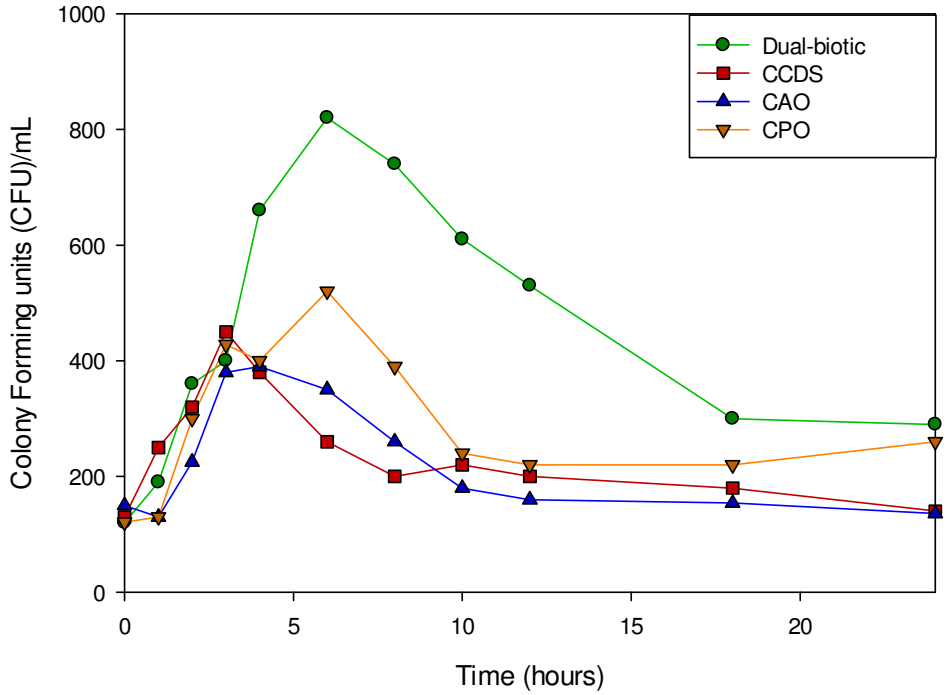


Figure 7.14: *Lactobacilli* release profiles determined through analysis of aspirated intestinal fluid samples onto *Lactobacillus*-specific agar. (CCDS= concomitant conventional delivery, CAO= conventional amoxicillin only, CPO= conventional probiotic only), n=5, SD≤43.3 in all cases.

7.4 Pharmacokinetic analysis

7.4.1 Amoxicillin non-compartmental and compartmental analysis

The results of the non-compartmental and compartmental pharmacokinetic analysis of amoxicillin release data are represented in Tables 7.2 and 7.3 and Figure 7.15. Evaluation of analysis revealed that amoxicillin release was best described by a one compartmental model with T_{lag} with the most favorable AIC and SBC values determined with this algorithm (Table 7.3). The most ideal sum of squares (SS) and standard error (SE) values were also determined with the one compartmental model with T_{lag} (SS= 0.0756; SE= 0.1375) when compared to the one compartmental model without T_{lag} (SS=2.8116; SE=0.6845) and two compartment model with T_{lag} (SS= 0.0752; SE=0.1583). Minimal SS and SE values are indicative of a lower fit detailing a low variability in data points. Evaluation of the R^2 values and regression residuals (R observed-predicted values), however, determined no distinct difference between the one compartmental model with T_{lag} and the two compartmental model with T_{lag} (0.9971 and 0.9975 respectively).

Pharmacokinetic parameters determined by the one compartmental model with T_{lag} depicted a T_{lag} of 0.8790 hours, a first order absorption rate constant of 0.5690/h, an elimination rate

constant of 0.4622/h, an elimination half-life of 1.4996 hours and a distribution half-life of 1.2181 hours. Additionally C_{max} was determined to be 3.3216µg/mL with T_{max} occurring at 2.7466 hours. The indicated first order absorption rate constant confirmed the rapid transition of the Dual-Biotic System into the intestine with rapid absorption thereafter taking place. The absorption of amoxicillin has been determined to be undertaken by a specialized transportation system in the intestine which explains its rapid quantification in plasma despite its poor liposolubility (Deslande *et al*., 1992). This transportation system was, however, not saturated due to the low loading dose of amoxicillin used. The absorption of amoxicillin has also been noted to be increased with the ingestion of water, which was *ad libitum* to all pigs utilized (Deslande *et al*., 1992). The effectiveness of the delivery system to provide the required concentration of amoxicillin to exert antimicrobial effects is also noted with the C_{max} being over the required levels as discussed in Section 7.3.4, with the MRT value being 3.9208, stating a lengthy period of time for the antibiotic to exert its time-dependent antimicrobial action. The rapid clearance of amoxicillin also occurs, primarily by renal excretion, with 60%-80% of the dose excreted unchanged. This can be attributed to the hydrophilic nature of amoxicillin. The elimination half-life was noted to be approximately 90 minutes, similar to the half-life of amoxicillin in pigs determined by Hernandez *et al*. (2005) of 82.5 minutes (SD= 22.7). The determined AUC_{0-t} and AUC_{0-inf} values also exhibited a minimal difference in amoxicillin concentration after the last observed value depicting rapid clearance of amoxicillin as well as the sensitivity of the quantification analysis. Evaluation of the volume of distribution (VD) determined a VD of approximately 30.61, well above the total volume of the pig, indicative of good drug penetration properties vital in the functioning of antibiotics (Schmitt-Hoffmann *et al*., 2006). Th results of the pharmacokinetic analysis provided important information on the gastric-emptying time of the Large White pigs utilized in addition to the rapid absorption and excretion of amoxicillin. The therapeutic antimicrobial effectiveness of the delivery system has also been noted with this analysis.

Table 7.2: Results of the non-compartmental analysis of amoxicillin release.

Parameter	Unit	Value
λ_z	1/hour	0.4441
$t_{1/2}$	Hour	1.5609
T_{max}	Hour	3.0000
C_{max}	µg/mL	3.2583
T_{lag}	Hour	0.0000
C_{last_obs}/C_{max}		0.0379
AUC $_{0\text{-}t}$	µg/mL*hour	17.1027
AUC $_{0\text{-}inf\_obs}$	µg/mL*hour	17.3810
AUC $_{0\text{-}t/0\text{-}inf\_obs}$		0.9840
AUMC $_{0\text{-}inf\_obs}$	µg/mL*hour^2	79.8300
MRT $_{0\text{-}inf\_obs}$	Hour	4.5930
Vz/F_{obs}	(mg)/(µg/mL)	32.3905
Cl/F_{obs}	(mg)/(µg/mL)/hour	14.3835

λ_z: *1st order rate constant of the terminal (log-linear) portion of the curve*
$t_{1/2}$: *Terminal phase elimination half-life.*
T_{max}: *Time at which the maximum amoxicillin concentration (C_{max}) is observed*
C_{max}: *Maximum observed amoxicillin concentration occurring at T_{max}*
T_{lag}: *Time taken for the amoxicillin to appear in systemic circulation following oral administration*
C_{last}: *The last quantifiable concentration following oral dosing*
$AUC_{0\text{-}t}$: *AUC from the dosing time to the last measurable amoxicillin concentration*
$AUC_{0\text{-}inf\ obs}$: *AUC from dosing time extrapolated to infinity based on the last observed amoxicillin concentration*
$AUMC_{0\text{-}inf\ obs}$: *Area under the first moment curve (AUMC) extrapolated to infinity based on the last observed amoxicillin concentration*
$MRT_{0\text{-}inf\ obs}$: *Mean residence time (MRT) extrapolated to infinity*
V_z/F_{obs}: *The observed volume of distribution based on the terminal phase, where F equals the fraction of the oral amoxicillin dose absorbed.*
Cl/F_{obs}: *The observed total body clearance for extravascular administration of amoxicillin.*

Table 7.3: Results of the compartmental analysis of amoxicillin release.

Parameter	Unit	Value	Diagnostics	Value
One compartment model without T_{lag}				
A	µg/mL	126.1074	r obs-pre	0.9165
k_a	1/hour	0.3749	SS	2.8116
k_{10}	1/hour	0.3535	R^2	0.9128
$t_{1/2ka}$	Hour	1.8489	SE	0.6845
$t_{1/2k10}$	Hour	1.9609	AIC	15.3038
V/F	(mg)/(µg/mL)	34.6920	SBC	15.8954
CL/F	(mg)/(µg/mL)/hour	12.2629		
T_{max}	Hour	2.7466		
C_{max}	µg/mL	2.7294		
AUC $_{0\text{-}t}$	µg/mL*hour	18.9968		
AUC $_{0\text{-}inf}$	µg/mL*hour	20.3867		
AUMC	µg/mL*hour^2	112.0530		
MRT	Hour	5.4964		
One compartment model with T_{lag}				
A	µg/mL	43.5078	r obs-pre	0.9971
k_a	1/hour	0.5690	SS	0.0756
k_{10}	1/hour	0.4622	R^2	0.9977
T_{lag}	Hour	0.8790	SE	0.1375

$t_{1/2ka}$	Hour	1.2181	AIC	-12.6570
$t_{1/2k10}$	Hour	1.4996	SBC	-12.3393
V/F	(mg)/(µg/mL)	30.6108		
CL/F	(mg)/(µg/mL)/hour	14.1490		
T_{max}	Hour	2.8254		
C_{max}	µg/mL	3.3216		
AUC $_{0-t}$	µg/mL*hour	17.3847		
AUC $_{0-inf}$	µg/mL*hour	17.6690		
AUMC	µg/mL*hour^2	69.2770		
MRT	hour	3.9208		
Two compartment model with T_{lag}				
A	µg/mL	11.3640	r obs-pre	0.9975
Alpha	1/hour	0.4595	SS	0.0752
B	µg/mL	38.1911	R^2	0.9977
Beta	1/hour	0.4709	SE	0.1583
k_a	1/hour	0.5620	AIC	-11.2915
T_{lag}	Hour	0.8792	SBC	-10.1081
k_{10}	1/hour	0.4680		
k_{12}	1/hour	5.22x10^{-5}		
k_{21}	1/hour	0.4624		
$t_{1/2Alpha}$	Hour	1.5084		
$t_{1/2Beta}$	Hour	1.4721		
$t_{1/2ka}$	Hour	1.233		
V/F	(mg)/(µg/mL)	30.2617		
CL/F	(mg)/(µg/mL)/hour	14.1617		
V2/F	(mg)/(µg/mL)	0.0034		
CL2/F	(mg)/(µg/mL)/hour	0.0016		
T_{max}	Hour	2.8265		
C_{max}	µg/mL	3.3210		
AUC $_{0-t}$	µg/mL*hour	17.3724		
AUC $_{0-inf}$	µg/mL*hour	17.6533		
AUMC	µg/mL*hour^2	69.1414		
MRT	Hour	3.0374		

A: *The zero time intercept associated with the alpha phase*

Alpha: *The initial phase of rapid decrease in plasma amoxicillin concentration due to distribution*

B: *The zero time intercept associated with the beta phase*

Beta: *The phase of gradual decrease in plasma amoxicillin concentration after the alpha phase due to metabolism and excretion*

k_a: *1st order absorption rate constant*

k_{10}: *Elimination rate constant*

$t_{1/2Ka}$: *Distribution half-life*

$t_{1/2k10}$: *Elimination half-life*

V/F: *The volume of distribution of the absorbed fraction of amoxicillin*

CL/F: *The observed total body clearance following extravascular administration of amoxicillin*

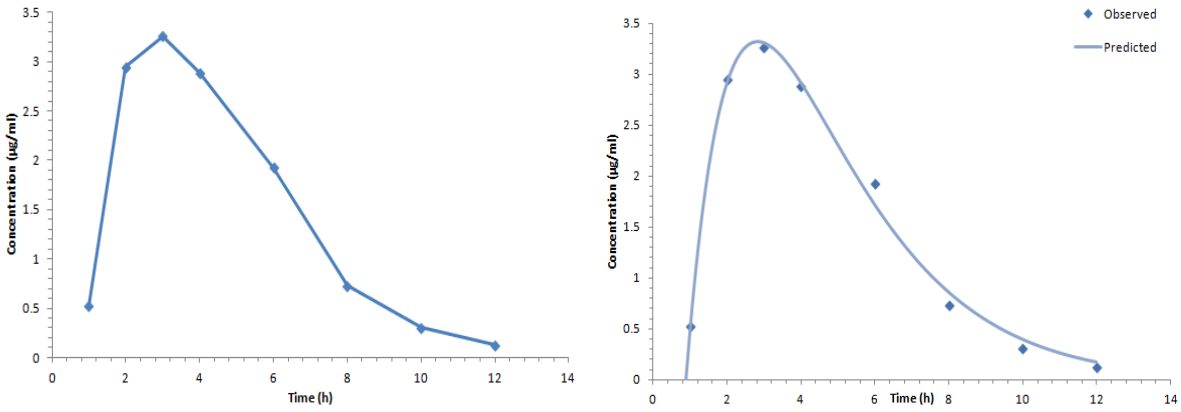


Figure 7.15: Graphical representations of (a) the non-compartmental and (b) compartmental pharmacokinetic analysis of amoxicillin release with observed and predicted values depicted.

7.4.2 *Lactobacillus* non-compartmental and compartmental analysis

The results of the non-compartmental and compartmental pharmacokinetic analysis of *L. acidophilus* release are represented in Tables 7.4 and 7.5 and Figure 7.16. Evaluation of analysis revealed that *L. acidophilus* release was best described by a one compartmental model with T_{lag} with the most favorable AIC and SBC values determined with this algorithm (Table 7.3). Further evaluation of the R^2 values determined no distinct difference between the one compartmental model with T_{lag}, one compartmental model without T_{lag} and the two compartmental model with T_{lag}. Whilst pharmacokinetic modeling is traditionally conducted to predict plasma concentrations and metabolic elimination via blood and urine aspiration, the one compartmental model with T_{lag} was utilized to predict the rate of increase in intestinal *L. acidophilus* count and the subsequent decline over a 24 hour period (Ravindran, 2011). Results of the analysis detailed a T_{lag} of 5.4409 hours, stating that the time required for an increase in intestinal *L. acidophilus* to be detected, a rate of *L. acidophilus* decrease (elimination rate constant) of 3.419/h and the time required for a 50% decrease in *L. acidophilus* counts of 2.9422 hours. Additionally, the C_{max} was determined to be 46357CFU/mL with T_{max} occurring at 6.2812 hours. The reproducibility of the model is of concern due to the intestinal fluid aspiration procedure utilized, therefore while providing an approximate T_{lag}, C_{max} and T_{max}, the model cannot be utilized further for the prediction of *L. acidophilus* counts.

Table 7.4: Results of the non-compartmental analysis of *L. acidophilus* release.

Parameter	Unit	Value
λ_z	1/hour	0.0561
$t_{1/2}$	Hour	12.3586
T_{max}	Hour	6.0000
C_{max}	CFU/mL	32284.0800
T_{lag}	Hour	0.0000
C_{last_obs}/C_{max}		0.0951
AUC_{0-t}	CFU/mL*hour	273824.0060
AUC_{0-inf_obs}	CFU/mL*hour	328542.5754
$AUC_{0-t/0-inf\_obs}$		0.8335
$AUMC_{0-inf\_obs}$	CFU/mL*hour^2	4825921.1310
MRT_{0-inf_obs}	Hour	14.6889

Table 7.5: Results of the compartmental analysis of *L. acidophilus* release.

Parameter	Unit	Value	Diagnostics	Value
One compartment model without T_{lag}				
A	CFU/mL	138854.5791	r obs-pre	0.9837
k_a	1/hour	0.5554	R^2	0.9845
k_{10}	1/hour	0.2168	AIC	126.6912
$t_{1/2ka}$	Hour	1.2480	SBC	126.5290
$t_{1/2k10}$	Hour	3.1977		
T_{max}	Hour	2.7784		
C_{max}	CFU/mL	46356.6788		
AUC_{0-t}	CFU/mL*hour	387034.5542		
AUC_{0-inf}	CFU/mL*hour	390559.5174		
AUMC	CFU/mL*hour^2	2504979.9090		
MRT	Hour	6.4138		
One compartment model with T_{lag}				
A	CFU/mL	44297.8065	r obs-pre	0.9839
k_a	1/hour	3.4193	R^2	0.9862
k_{10}	1/hour	0.2356	AIC	110.7405
T_{lag}	Hour	5.4409	SBC	109.9075
$t_{1/2ka}$	Hour	0.2027		
$t_{1/2k10}$	Hour	2.9422		
T_{max}	Hour	6.2812		
C_{max}	CFU/mL	33838.3817		
AUC_{0-t}	CFU/mL*hour	174418.8696		
AUC_{0-inf}	CFU/mL*hour	175077.6060		
AUMC	CFU/mL*hour^2	794363.0458		
MRT	Hour	4.5372		
Two compartment model with T_{lag}				
A	CFU/mL	52378.3187	r obs-pre	0.9915

Alpha	1/hour	0.3412	R^2	0.9924
B	CFU/mL	3157.1485	AIC	127.7192
Beta	1/hour	1x 10^{-6}	SBC	127.3946
k_a	1/hour	2.4400		
T_{lag}	Hour	5.4400		
k_{10}	1/hour	1.52699x10^{-5}		
k_{12}	1/hour	0.3188		
k_{21}	1/hour	0.0223		
$t_{1/2Alpha}$	Hour	2.0315		
$t_{1/2Beta}$	Hour	693147.1806		
$t_{1/2ka}$	Hour	0.2841		
T_{max}	Hour	6.4052		
C_{max}	CFU/mL	35569.2191		
AUC $_{0-t}$	CFU/mL*hour	206481.4795		
AUC $_{0-inf}$	CFU/mL*hour	3157279295.0000		
AUMC	CFU/mL*hour^2	3.15715x 10^{15}		
MRT	Hour	999953.1468		

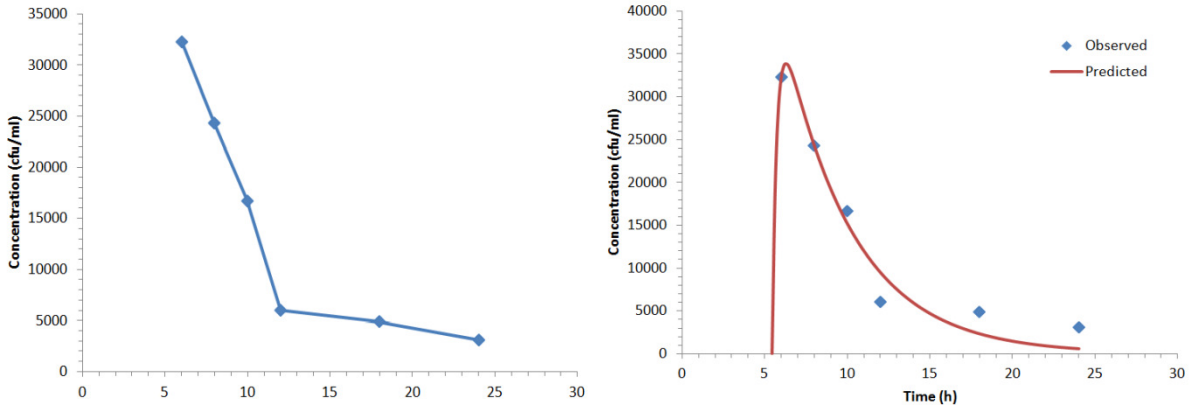


Figure 7.16: Graphical representations of (a) the non-compartmental and (b) compartmental pharmacokinetic analysis of *L. acidophilus* release with observed and predicted values depicted.

7.4.3 Deconvolution and establishment of an *in vitro-in vivo* correlation

A Level A IVIVC was developed in the evaluation of amoxicillin release, which uses all the data provided by the *in vitro* dissolution and *in vivo* absorption curves and is based directly upon each of the assayed time points analyzed (Amann *et al*., 2010). The amount of amoxicillin absorbed following administration of the Dual-Biotic System was therefore calculated by the Wagner Nelson method using the linear trapezoidal rule. For the construction of a Level A IVIVC, the percentage of drug absorbed up to time *t* was plotted versus the amount of drug released *in vitro*.

The pharmacokinetic conditions derived from this investigation conforms to the requirements of deconvolution through convolution, as amoxicillin release from the Dual-Biotic System was determined to be through a one-compartmental pharmacokinetic model with $T_{lag.}$ Deconvolution of *in vivo* plasma amoxicillin concentration-time data against *in vitro* release data provided the percentage drug release of the Dual-Biotic System in the respective analyses (Figure 7.17).

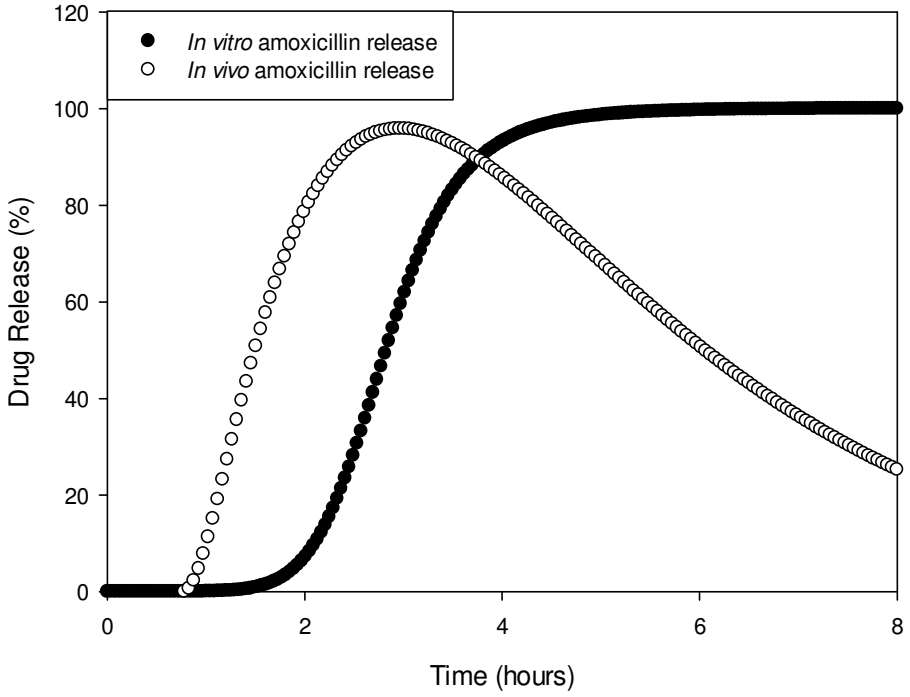


Figure 7.17: Predicted drug release profiles of the *in vitro* and *in vivo* amoxicillin release from the Dual-Biotic System obtained using deconvolution analysis.

Level A IVIVC analysis yielded a levy plot with a R^2 value of 0.9696 (Figure 7.18). Thus *in vitro* data was predictive of *in vivo* data with 96.7% accuracy. Whilst the plots determined were not directly superimposible, based on the qualitative patterns of the fit, a Level A correlation was, however, approached. The result seen, where a faster *in vivo* absorption was noted when compared to the *in vitro* dissolution profile, can be attributed to the gastrointestinal transit time (between simulated gastric and intestinal conditions) utilized *in vitro* that did not accurately correlate to transition and release *in vivo*, which occurred at a much quicker rate. It was also noted in Chapter 6, Section 6.3.9 and Section 7.3.4 that the polymer coating utilized in the Dual-Biotic System dissolved at a much quicker rate during *ex vivo* release studies, when compared to the *in vitro* analysis. The high permeability of amoxicillin could also be a contributing factor with a marginally steeper gradient in the *in vivo* profile noted, when compared to *in vitro* profile in Figure 7.17. This is in correlation with the

results determined in Chapter 6, Section 6.3.10, which determined the high permeability of the amoxicillin utilized in the Dual-Biotic System. The prediction errors (PE) for C_{max} and $AUC_{0-\infty}$ for this Level A IVIVC analysis were calculated to be 4.15 and 13.15% respectively, marginally more than the specified 10% PE for a good Level A correlation, but still within the 15% requirement for an individual formulation.

A Level A IVIVC could not be achieved for the evaluation of *L. acidophilus* release data. This is due to the differences between the *in vitro* and *in vivo* sampling procedures used, where a controlled *L. acidophilus* release profile was constructed *in vitro* (Figure 5.14), which could not be reproduced *in vivo* (Figure 7.12). The *in vivo* profile generated, by utilizing the placebo group as the reference CFUs, ascertained increases in *L. acidophilus* CFUs at the sampling point in the intestinal ileum, as opposed to *L. acidophilus* counts across the entire small intestine which the *in vitro* analysis allowed. This resulted in a successful deconvolution of *in vitro* data with predicted values determined that could not be correlated to *in vivo* data where a spike in *L. acidophilus* CFUs was determined rather than a controlled release profile, even though it may have taken place. This phenomenon can be overcome through the use of bacterial fluorescent labeling with release tracking undertaken (Marteau and Shanahan, 2003). This could not, however, be performed in this *in vivo* analysis due to the constraints associated with prolonged anesthesia of the pig subjects over the analysis period, in addition to the undesirable physiological effects associated with inhaled anesthesia such as a delayed gastric emptying and gastrointestinal transit (Anderson *et al*., 2002). This would ultimately lead to a release profile that could not be correlated to the conscious pigs utilized in this study.

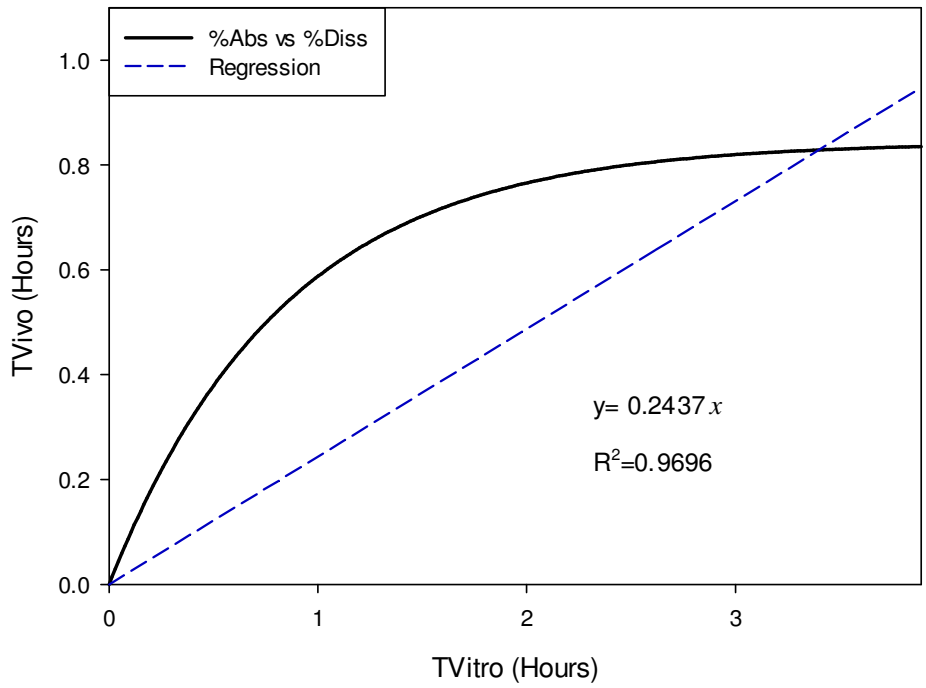


Figure 7.18: A Levy plot depicting the relationship between the percentage of amoxicillin released *in vitro* and the percentage of amoxicillin released *in vivo*.

7.5 Concluding Remarks

The success of the Dual-Biotic System was determined through *in vivo* analysis in a Large White pig model. Site-specific delivery was achieved through the use of the gastro-resistant capsule coating solution with the formulated delayed release mechanism ensuring protection of the administered *L. acidophilus* probiotic against the bactericidal effects of amoxicillin. The design and surgical implantation of the intestinal cannula was also successful with intestinal fluid aspirated as required. Evaluation of the Dual-Biotic System therefore accurately proved that through this formulation, the effects of antibiotic-associated diarrhea can be prevented, ultimately removing the requirement for patients to take these two formulations apart, increasing the therapeutic desirability of antibiotic therapy.

CHAPTER 8

CONCLUSION AND RECOMMENDATIONS

8.1 Conclusions

The development of novel delivery systems by integrating a multitude of scientific fields has been shown through this study to challenge current methodology and clinical practices with the aim of improving therapeutic benefits for patients. With the controversy associated with intestinal flora supplementation, the current agreement is that probiotic products provide vital health benefits to a patient and if administered in the appropriate manner, will exert the therapeutic benefits intended. The Dual-Biotic System formulated in this study has been developed and extensively analyzed to overcome the issues encountered in the concomitant delivery of probiotics with antibiotics. This has been undertaken by developing an optimized denatured crosslinked biopolymeric carrier matrix for the protection and delivery of probiotic bacteria that are often deactivated during formulation processes. The ovalbumin utilized also has the added benefit of providing growth media to the probiotic bacteria, encouraging bacterial growth and subsequent adhesion to the intestinal wall.

The delayed release system utilizing glyceryl-monostereate was effective in the protection of the encapsulated ovalbumin matrix while having the added advantage of loose particle aggregation which is beneficial with multiparticulate systems. Finally the Dual-Biotic System was proven effective through advanced *in vitro*, *ex vivo* and *in vivo* analyses. *In vitro* release studies performed determined that the Dual-Biotic System successfully protected the administered probiotic bacteria against the co-administered antibiotic. *In vitro* characterization was further utilized to ascertain the thermal, structural, physiochemical and physicomechanical properties of the various systems developed in this study, with matrix integrity maintained and ensured at all times through crystallinity and infra-red spectroscopy analyses. *Ex vivo* studies were undertaken to determine the bacterial adhesion properties of *L. acidophilus* in comparison with a commercial probiotic formulation. Results determined a high adhesion potential in correlation with the commercial product but, more importantly detailed the inhibition of known pathogenic bacteria from adhering to the intestinal wall.

In vivo analysis utilizing Large White pigs further sort to improve on the current methodology for characterization of probiotic products. This was undertaken by developing a stable

cannula for the aspiration of intestinal fluid that was utilized for the quantification of *Lactobacilli* in intestinal media. Results of this analysis detailed the effectiveness of the Dual-Biotic System in contrast to the commercially available probiotic and antibiotic systems, which detailed the drastic decrease of probiotic bacteria when the respective formulations were administered concomitantly. Pharmacokinetic modeling of *in vivo* results determined that amoxicillin and probiotic release were best described by a one compartmental model with lag with *in vitro-in vivo* correlation analysis ascertaining a good Level A correlation with respect to amoxicillin release.

The use of luminescence for bacterial quantification was also evaluated as a part of this study. Luminescence proved advantageous for the quantification of probiotic bacteria in solution and was correlated with UV spectroscopy for the respective release profiles. This confirmed the utilization of both analytical techniques in probiotic characterization and improved on current microbial methodology that is less cost-effective and more time consuming. Microbial incubation, however, as described in this study can be utilized for the confirmation of *in vivo* results where a possibility of microbial contamination by native intestinal and colonic flora exists.

This thesis therefore focused on the development to preclinical analysis of a Dual-Biotic System for the concurrent administration of antibiotics and probiotics. Results have determined formulation efficacy and ensured probiotic viability at all stages of this study including *in vitro, ex vivo* and *in vivo* analyses. Novel and innovative drug delivery designs and techniques have been further developed which seeks to improve on current methodology for the characterization of probiotic formulations which will ultimately lead to greater clinical outcomes for patients. In summary, through this research study, a novel Dual-Biotic System has been developed and extensively analyzed, with the possibility of this system clinically enhancing therapeutic outcomes associated with antibiotic therapy, which would ultimately benefit a large proportion of patients in the world.

8.2 Recommendations

Throughout the development and analysis of the Dual-Biotic System, many issues concerning probiotic therapy and the concomitant administration of probiotics with antibiotics were encountered and overcome. Whilst the success and efficacy of the Dual-Biotic System has been detailed up to a preclinical perspective, various factors will have to be considered for the advancement of this delivery system to the public for large scale use. Firstly, whilst the Dual-Biotic System is relatively cheap to formulate and can be cost-effectively utilized on a large scale, the variety of steps required for the final system would possibly require dedicated machinery for large scale manufacture. The use of large scale compactors and blenders would, however, prove beneficial as opposed to the formulation of tablet slugs for the development of the Dual-Biotic System. Pre-coated capsules would also prove to be cost-effective as opposed to the individual capsule coating performed in this study. The use of ovalbumin as the carrier matrix also serves as a possible obstacle with the reliance on chicken eggs that can be impacted by both ethical and procurement issues. More research can therefore be conducted for the development of possible probiotic carrier matrices that can be modified for the requirements of a delivery system. These systems could include alginate, chitosan, hydroxypropyl methylcellulose phthalate and high amylose starch which as noted in Chapter 2 have been effective carriers for probiotics. A more detailed investigation into the use of other common cost-effective polymers for probiotic delivery could also be undertaken. Additionally, as stated in Chapter 1, the issue of egg allergy due to ovalbumin is also of concern despite research determining a decrease in allergenicity with modification of the protein structure. Allergenicity tests can therefore be undertaken prior to clinical evaluation of the Dual-Biotic System.

With the success of the Dual-Biotic System detailed in this study, the adaptability of the system can be evaluated. *L. acidophilus* was utilized as the model probiotic due to its wide utilization in probiotic formulations and ability to withstand gastric conditions. Other bacterial species however, can be incorporated into the Dual-Biotic System with different probiotic combinations possible, provided functional health benefits are maintained. The documented biocompatibility of the various components of the Dual-Biotic System is also beneficial as it removes the requirement for extensive histopathological studies in the future. The delayed release system can also be utilized in future for the site specific delivery of probiotics similar to the manner of the BMTTS described in Appendix C, where different ratios of GMS can be utilized and combined into a single delivery system. With the wide range of antibiotics available, the adaptability of the Dual-Biotic System with other antibiotics can also be determined with the optimized and delayed release systems modified accordingly to the

pharmacokinetics of the antibiotic used. This therefore leaves the development of further Dual-Biotic Systems, or 'Antrobiotics', a distinct possibility.

Through the various innovative analyses performed in this study, a great number of modifications were undoubtedly undertaken in the conduction of the *in vivo* analysis. The designed and manufactured intestinal cannula was proven to be an effective technique for the aspiration of intestinal fluid. A few potential obstacles were, however, noted during practical use of the cannula. One such issue was the complete assembly of the cannula prior to surgical implantation. This was a hindrance during the surgical procedure as larger extrusion ports were required with suturing under the cannula collar required to prevent removal of the cannula during the course of the study. It will therefore be beneficial to place the collar onto the cannula body during the surgical implantation procedure, using surgical glue rather than assembling the cannula prior to surgery. The intestinal cannula itself could be modified for greater stability. An added collar placed under the skin, as depicted in Figure 8.1, could be included in the assembly to allow for a more rigid, less removable, cannula structure to be formed. This would allow for utilization of the implanted cannula for a longer period of time.

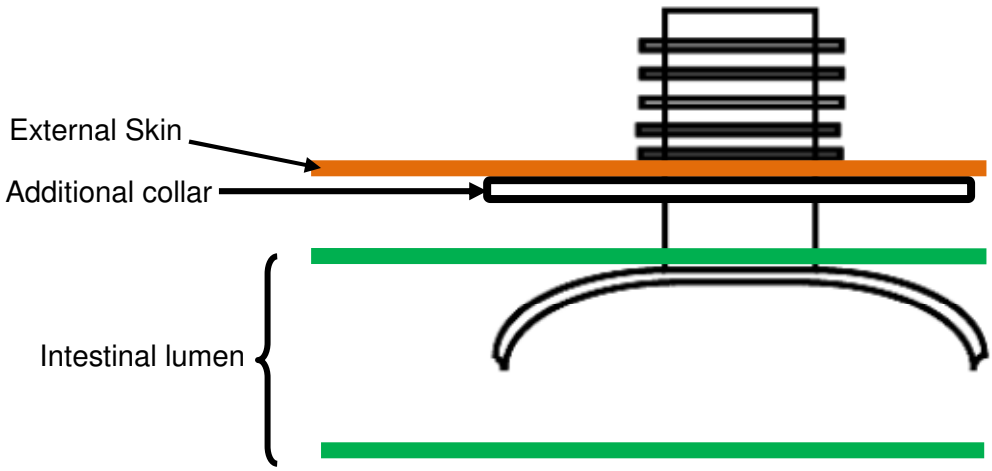


Figure 8.1: Schematic of the proposed modified stable intestinal cannula.

Further improvement on quantification and modeling of probiotic release analyses can also be undertaken. Whilst the luminescence protocol utilized in this study provides crucial data on the viability of cells, further improvements could be made in the future to provide more detail information on the status of the cells in solution. Advancements in probiotic release data modeling could also be undertaken where a model could be developed that would cater

for the specialized delivery of viable actives such as probiotic bacteria. This would allow for a more predicative probiotic release *in vivo* utilizing *in vitro* data. In addition, the bacteria incorporated into the Dual-Biotic System can be labeled and tracked in an animal model, this not being possible in this study due to the impracticality to maintain a large number of pigs under anesthesia over the *in vivo* release analysis. Bacterial marking and tracking can be advantageous in the construction of more accurate probiotic release profiles (Marteau and Shanahan, 2003).

The development of the Dual-Biotic System for concurrent antibiotic and probiotic delivery has provided the possibility of simple single delivery system that would remove the issue of patient compliance, whilst still ensuring therapeutic efficacy for a patient. The results of this study can further be adapted to various other systems that require separate release mechanisms, with the innovative techniques utilized as a possible alternative to current methods for probiotic characterization. The research generated in this study will therefore provide important concepts and techniques that can be adapted to various other fields of research in addition to pharmaceutical drug delivery. This will ultimately contribute to the advancement of probiotic and antimicrobial research in South Africa, leading to an increase in research outputs, in addition to innovative and novel research projects.

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APPENDICES

APPENDIX A1

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Review Article

A Review of the Advancements in Probiotic Delivery: Conventional *vs.* Non-conventional Formulations for Intestinal Flora Supplementation

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Abstract. Probiotic delivery systems are widely used nutraceutical products for the supplementation of natural intestinal flora. These delivery systems vary greatly in effectiveness to exert health benefits for a patient. Probiotic delivery systems can be categorized into conventional, pharmaceutical formulations, and non-conventional, mainly commercial food-based, products. The degree of health benefits provided by these probiotic formulations varies in their ability to deliver viable, functional bacteria in large enough numbers (effectiveness), to provide protection against the harsh effects of the gastric environment and intestinal bile (*in vivo* protection), and to survive formulation processes (viability). This review discusses the effectiveness of these probiotic delivery systems to deliver viable functional bacteria focusing on the ability to protect the encapsulated probiotics during formulation process as well as against harsh physiological conditions through formulation enhancements using coatings and polymer enhancements. A brief overview on the health benefits of probiotics, current formulation, patient and legal issues facing probiotic delivery, and possible recommendations for the enhanced delivery of probiotic bacteria are also provided. Newer advanced *in vitro* analyses that can accurately determine the effectiveness of a probiotic formulation are also discussed with an ideal probiotic delivery system hypothesized through a combination of the two probiotic delivery systems described.

KEY WORDS: conventional and non-conventional formulations; drug delivery systems design; intestinal flora; nutraceutical products; probiotics.

INTRODUCTION

The Need for Intestinal Flora Supplementation

Probiotic bacteria are living supplementary organisms that have been shown to provide beneficial health effects to the host by replenishing natural gastrointestinal flora (1–5). The human intestinal system is thought to contain over 500 microbial species and approximately 10^{14} functional bacterial cells and also include fungi, yeasts, viruses, and protozoa. Some bacteria, such as *Streptococci* and *Staphylococci* spp., are known to cause infectious diseases in humans (6–9). These organisms are usually depleted for various reasons such as stress, infection, antibiotic use, and environmental factors. Other patients that require probiotic therapy are babies born of caesarean section and do not gain the bacteria it would naturally while travelling down the birth canal (9). Probiotic supplementation in these patients has been shown to be vital in the prevention of potentially fatal, pathological infections (10–12). A full representation of the microbial content of the human gastro-intestinal system can be found in Fig. 1.

Probiotic bacteria have also been widely recognized to have other health benefits to the patient such as effects on immunological functions, aiding in digestion, as well as protection against pathogenic bacteria such as *Salmonella typhimurium*, *Helicobacter pylori*, and *Escherichia coli* (12,14–17). Other functions of probiotics include improvement of lactose intolerance, decreasing cholesterol levels, treatment of Crohn's disease, ulcerative colitis, IBS, and replenishment of intestinal flora after antibiotic therapy to prevent antibiotic-induced diarrhea (18–23). Intestinal flora are also important in entero-hepatic recycling which results in the metabolizing of a variety of drugs. Probiotics, by definition, should adhere to the intestinal cells, not promote or encourage antibiotic resistance, not as themselves be pathogenic in nature, and must be able to co-aggregate as part of the natural gut flora (12,24). The degree at which the functional benefits are received are dependent on the type of bacteria delivered as well as the number of viable bacteria that is delivered to the gastro-intestinal system (25,26). A basic schematic diagram on the properties of an ideal probiotic can be found in Fig. 2.

Probiotic Formulations

The use of intestinal flora supplementation can be dated back decades with the first documented article of bacterial supplementation published in the early twentieth century by

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APPENDIX A2

A gastro-resistant ovalbumin mini-tablet-in-tablet system for the delivery of *Lactobacillus acidophilus* probiotic to simulated human intestinal and colon conditions

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Abstract

Objectives

Ovalbumin has been noted for its various pharmaceutical applications. This study focuses on the use of denatured ovalbumin for the delivery of probiotic bacteria (*Lactobacillus acidophilus*) to simulated human intestinal and colon conditions through the use of a gastro-resistant bi-layered mini-tablet-in-tablet system (BMTTS).

Methods

The formulated BMTTS consists of a gastro-resistant ovalbumin mini-tablet containing *Lactobacillus acidophilus* suspended in lactose for exposure in gastric media but targeted delivery in the intestinal system with another mini-tablet suspended in eudragit S100 for targeted colonic delivery. Luminescence has been utilized to determine the number of viable bacterial cells required to exert functional health benefits as well as to determine all probiotic release profiles. Analyses were conducted to determine the mechanism of probiotic release from the ovalbumin mini-tablet using UV spectroscopy and mathematical and molecular modelling through use of the Hopfenberg model and molecular docking studies respectively.

Key findings

Results of this study have shown that the ovalbumin BMTTS is a successful carrier-system *in vitro* for the delivery of *Lactobacillus acidophilus* to Simulated Human Intestinal and Colon conditions. Further molecular docking studies carried out to depict the interaction and binding positions inherent to the Ovalbumin–Pancreatic Trypsin interaction Complex (OPTiC) indicates the possible enzymatic degradation of ovalbumin leading to the release of the probiotic from the protein matrices in intestinal as opposed to gastric conditions.

Conclusions

Ovalbumin through this study has been proven to be effective in the protection and delivering of probiotic bacteria. Through the use of molecular docking analysis, pancreatin has been shown to exert a significant effect on the release of probiotic bacteria from the ovalbumin matrix. The formulated BMTTS has been proven *in vitro* to successfully deliver probiotic bacteria to simulated human intestinal and colonic conditions.

Keywords: Gastro-resistant, Intestinal and Colon Targeting, *Lactobacillus acidophilus*, Mini-tablet-in-tablet, Ovalbumin, Probiotic bacteria.

A modified MRS agar for the selective enumeration of *Lactobacilli* in porcine intestinal fluid

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Significance and impact of the study

Intestinal flora is comprised of a large number of bacteria, fungi and viruses. This wide diversity of microbiota often results in the requirement of specialized media or protocols for the isolation of specific species. *Lactobacilli* are one of the bacterial species found in natural intestinal flora and are commonly the active ingredient of many probiotic products. This study provides a simple, cost-effective modification of MRS media for the selective enumeration of *Lactobacilli* in porcine intestinal fluid. This modified media allows for the quantification of *Lactobacilli* used in probiotic products which can accurately determine formulation efficacy during *in vivo* studies.

Abstract

The isolation and quantification of *Lactobacilli* from intestinal fluid can be advantageous in the determination of probiotic formulation efficacy. This, however, often requires various agar media and sub-culturing techniques prior to quantification of the bacteria which leads to an

A Novel Oral Multi-Particulate System for the Concurrent Delivery of Amoxicillin and *Lactobacillus acidophilus*

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ABSTRACT

Aims A site-specific delayed release concurrent delivery system for amoxicillin and probiotic *Lactobacillus acidophilus* has been prepared and evaluated in this study in addition to the bacterial adhesion properties of *L. acidophilus*. **Materials and Methods** Statistical optimization of a crosslinked denatured ovalbumin matrix containing *L. acidophilus* was achieved utilizing a Box-Behnken experimental design prior to encapsulation with glyceryl-monostereate. Adhesion studies included exposure of excised porcine intestinal mucosa to *L.acidophilus*, *Clostridium difficile* and *Escherichia coli*. **Results** Evaluation of the concurrent delivery system detailed its success in protecting *L. acidophilus* probiotic against the bactericidal effects of amoxicillin. Adhesion analyses conducted noted the high adhesion potential of *L. acidophilus* as well as its protective properties against *C. difficile* and *E. coli*. **Conclusions and Future Perspective** The formulated system has shown to be effective for the concurrent delivery of *L. acidophilus* and amoxicillin. With the current focus of microbiome research being the identification, classification and utilization of functional healthy bacteria in humans, as outlined in the Human Microbiome Project, this study will provide further insight into the development of novel probiotic delivery systems.

KEYWORDS

Antibiotic and Probiotic Therapy; *Lactobacillus acidophilus*; Concurrent Delivery; Crosslinked Protein System; Accelerated Stability Studies; Bacterial Adhesion; Bacterial Fluorescent Imaging.

APPENDIX B1

A multi-particulate system for the concurrent delivery of amoxicillin antibiotic and *Lactobacillus acidophilus* probiotic

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Abstract

The aim of the study was to develop a novel multi-particulate system for the concurrent delivery of amoxicillin antibiotic and *Lactobacillus acidophilus* probiotic. The intended delivery system was developed to overcome the current issue affecting probiotic therapy which is killing of the probiotic bacteria due to the inappropriate administration of probiotics in relation with antibiotics leading to issues such as antibiotic-associated diarrhoea. The delivery system was developed using cultured *Lactobacillus acidophilus* bacteria that were freeze-dried into powder and thereafter quantified. Using a natural polymer of ovalbumin which is commonly found in egg albumin, the probiotic bacteria were suspended in the ovalbumin, freeze-dried, compressed into tablet slugs and formed into granules. The granules were encapsulated using a delayed-release polymer and thereafter placed in a gastro-resistant hard gelatine capsule intended for site-specific delivery of the formulation to the small intestine. Statistical software was used to determine the ideal quantities of ingredients for maximum delivery of probiotic bacteria prior to the next dose of the antibiotic and probiotic formulation. Results of this study showed that *in vitro*, the developed dosage form successfully delayed the release of *Lactobacillus acidophilus* probiotic by two hours to prevent the delivered antibiotic from coming into contact with the delivered probiotic. This was also successfully delivered in a gastro-resistant capsule that allowed for site-specific delivery of the formulation overcoming the issue of varying gastrointestinal transit times in multi-particulate systems.

APPENDIX B2

Fabrication, Statistical Optimization and Characterization of a Multi-particulate System for the Concurrent Delivery of Antibiotics and Probiotics

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Purpose:
Antibiotic therapy has proven to be vital in the treatment of life-threatening conditions. However, unwanted side effects such as intestinal flora destruction and the occurrence of antibiotic-associated diarrhoea have been documented. Probiotics have now become an effective adjunctive in the prevention of antibiotic-associated diarrhoea however incorrect administration with relation to antibiotics as well as lack of patient compliance often results in the failure of probiotic formulations. A site-specific delayed-release concurrent delivery system for antibiotics and probiotics (CDSAP) based on ovalbumin has been formulated, optimized and analysed in this study to overcome this issue. Upon optimization, a delayed release erodible polymer system was utilized to prevent physical contact between the administered antibiotic and probiotic bacteria.

Methods:
Statistical Optimization of the Functional Ovalbumin System: A Box-Behnken experimental design was employed to achieve an optimized ovalbumin formulation. Ovalbumin concentration, cross-linker percentage and buffer pH were taken as variables and their upper and lower limits were determined through *in vitro* probiotic release analysis in addition to probiotic viability. Optimization parameters included a targeted maximum probiotic release time of four hours to allow for maximum health benefits across the intestinal system prior to the next antibiotic dose as well as formulation properties such as granule flow and compressibility. All release profiles and viability assays were conducted using a luminescence protocol that accurately determined the number of probiotic cells in solution through bacterial ATP quantification.
Formulation of the Delayed-Release CDSAP System: The multi-particulate CDSAP system was prepared from the optimized ovalbumin formulation using the dry granulation method through compression of the ovalbumin containing probiotic into tablet slugs and subsequent breaking down into granules. To obtain the delayed release CDSAP system, an erodible polymer was used with the ovalbumin in the weight ratio of 1.94:1 achieving a delay of two hours.

Results:
The optimized formulation was determined to consist of 0.8868mL ovalbumin, 0.0058% genipin and a buffer pH of 6.9929. *In vitro* probiotic release profiles have shown the success of the formulated concurrent delivery system with the possibility of this delivery system to be used widely for the prevention of conditions associated with intestinal flora destruction due to antibiotic therapy. The formulated optimized and CDSAP systems furthermore showed excellent flow and compressibility properties in accordance with the optimization protocol used. Ovalbumin, in this study, was determined to be a suitable carrier system for probiotic bacteria with the additional benefit of providing growth media for the bacteria upon release from the ovalbumin matrix.

APPENDIX B3

In vivo analysis of a concurrent delivery system for antibiotics and probiotics in a Large White pig model

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A concurrent delivery system designed and developed to overcome the therapeutic issue of the concomitant administration of probiotic products with antibiotic formulations has been evaluated in this study. In such concomitant administration, there is often the destruction of the probiotic bacteria making the patient vulnerable to unwanted side-effects such as antibiotic-associated diarrhoea. The developed concurrent delivery system comprises of antibiotic and probiotic loaded multiparticulates equipped with individual component release at different time intervals. The probiotic component is tailored to exhibit a delayed release which allows for the absorption of the antibiotic prior to the release of the probiotic bacteria. The key component of the delayed release system is optimized ovalbumin granules encapsulated in an erodible polymer matrix of glycerylmonostereate at a ratio of 1:1.94. The concurrent delivery system was evaluated in a Large White pig model surgically implanted with a specially designed intestinal cannula into the ileum for intestinal fluid aspiration and an intra-jugular catheter for plasma sampling. *In vivo* analysis determined the effectiveness of the concurrent delivery system in protecting *Lactobacillus acidophilus* probiotic against the bactericidal effects of amoxicillin. Evaluation of aspirated samples revealed maximum amoxicillin plasma levels at 3 hours post administration and maximum probiotic cell counts occurring after 6 hours. A Level A *in vitro-in vivo* correlation was also established with a 99.6% predictability of amoxicillin release.

APPENDIX C

A BI-LAYERED MINITABLET-IN-TABLET SYSTEM FOR THE DELIVERY OF *LACTOBACILLUS ACIDOPHILUS* PROBIOTIC TO SIMULATED HUMAN INTESTINAL AND COLON CONDITIONS

C.1 Introduction

Ovalbumin has been proven to be effective in the protection and delivery of *L. acidophilus* probiotic. With the granule system chosen to be advanced into the formulated Dual-Biotic System, the ovalbumin minitablet was advanced into a bi-layered-minitablet-in-tablet system (BMTTS) that would provide for the site specific delivery of *L. acidophilus* to simulated human intestinal and colon conditions. The outer layer chosen for probiotic delivery to the small intestine was lactose. Lactose is a commonly used binding and filling agent in pharmaceutical applications that readily dissolves in gastric conditions. This was, however, acceptable due to the gastro-resistant properties of the formulated ovalbumin minitablet that allowed for tablet exposure in the gastric media but released probiotic bacteria in conditions associated with the small intestine. Whilst it would be acceptable to simply administer the minitablet system without the addition of lactose for site-specific delivery in the small intestine, the lactose was required for maintenance of tablet shape integrity to allow for the addition of a second tablet layer for the site-specific delivery of probiotics to the colon. For the delivery of probiotics to colonic conditions eudragit S100 was chosen. Eudragit S100 is a pH-sensitive polymer that is soluble in conditions associated with the colon and was therefore utilized as the polymer for targeted colonic delivery (Chawla *et al.*, 2012). The BMTTS was formulated as depicted in Figure C.1. This appendix therefore provides the formulation and characterization of a BMTTS for the site-specific delivery of probiotic bacteria to simulated human intestinal and colon conditions utilizing the formulated ovalbumin minitablet analyzed in Chapter 3.

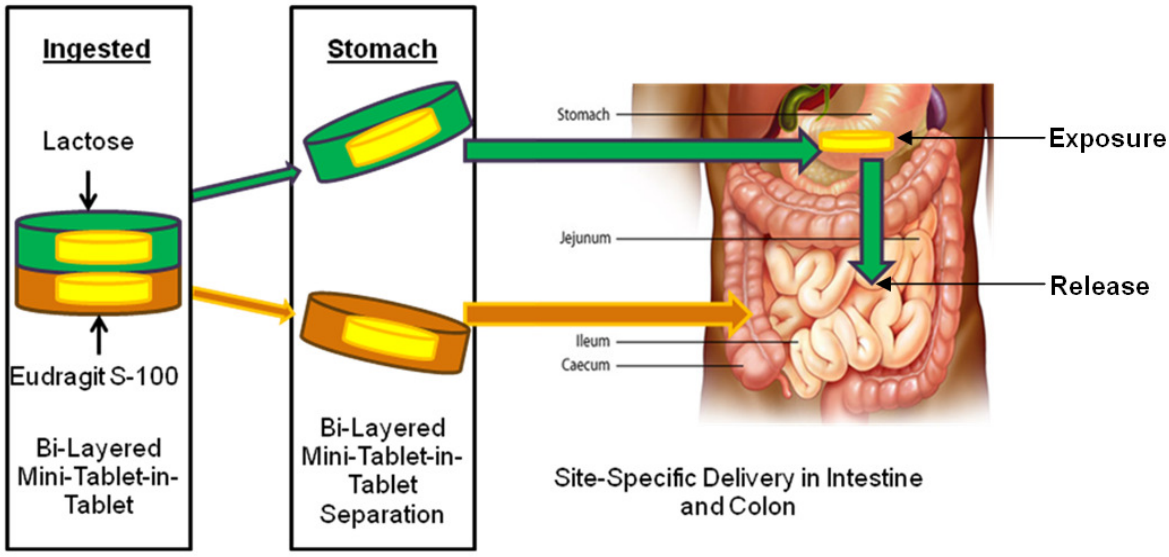

Figure C.1: A diagrammatic representation of the BMTTS for intestinal and colonic delivery of *L. acidophilus*.

C.2 Materials and Methods

C.2.1 Materials

Lactobacillus acidophilus ATCC 314 was purchased from Davies Diagnostics (Randburg, Gauteng, South Africa). Raw egg ovalbumin was obtained from commercial chicken eggs (Nulaid®, Grade 1, Grain Fed) purchased from Pick n Pay® Stores (Johannesburg, Gauteng, South Africa). Purified ovalbumin derived from chicken eggs, pancreatin from porcine pancreas and pepsin from porcine gastric mucosa were purchased from Sigma-Aldrich (St Louis, MO, USA). Skimmed Milk was purchased from Clover® (Roodepoort, South Africa). MRS Broth, Thioglycolate Broth, Thioglycolate Agar and Tryptone Soya Agar were purchased from Oxoid (Hampshire, UK). BacTitre-Glo microbial cell viability assay was purchased from Promega (Madison, WI, USA). Eudragit S100 was purchased from Rohm Pharma Polymers (Darmstadt, Germany). All other chemicals were of analytical grade and used as received.

C.2.2 Isolation and stock maintenance of *L. acidophilus* cells

L. acidophilus probiotic was prepared and quantified using the method as outlined in Chapter 3, Section 3.2.2.

C.2.3 Preparation of a gastro-resistant BMTTS for the delivery of *L. acidophilus* to simulated small intestinal and colon conditions

The gastro-resistant ovalbumin minitablet was formulated as stated in Chapter 3, Section 3.2.6.1. The BMTTS was formulated using a minitablet-in-tablet compression technique at a force of 1 ton utilizing 300mg of lactose and eudragit S100 for the outer tablet layers for intestinal and colonic delivery respectively. Finally the two layers were compressed to form a single BMTTS.

C.2.4 Matrix hardness determination of BMTTS

The matrix hardness of the individual layers of the BMTTS was determined utilizing a calibrated Texture Analyzer as stated in Chapter 3, Section 3.2.6.3. Matrix hardness of the BMTTS is represented by conversion to the Brinell Hardness Number using Equation 3.1.

C.2.5 Friability analysis of BMTTS

The friability of the individual layers of the BMTTS was determined using a Friabilator rotating at 25rpm for 4 minutes representing the 100 rotations required for friability determination as required by USP specification.

C.2.6 *In vitro* probiotic release analysis of BMTTS

In vitro probiotic release analysis was conducted utilizing the method previously stated in Chapter 3, Section 3.2.6.7 modified for minitablet transition through the human GIT. Briefly, the formulated BMTTS was placed in simulated human gastric conditions for 2 hours, in intestinal conditions for a further 4 hours and thereafter in colonic conditions (USP dissolution apparatus containing 900mL PBS with rat faecal content at a pH of 7.4 rotating at 50rpm) for 8 hours. Samples (1mL) were withdrawn at hourly intervals and evaluated using the luminescence protocol stated in Chapter 3, Section 3.2.2.2. Fresh buffer (1mL) was added to the dissolution media after sample aspiration to maintain physiological sink conditions.

C.3 Results and Discussion

C.3.1 Matrix hardness of the BMTTS

The BHN of the lactose layer of the BMTTS was calculated to be 2.447 with the BHN value of the eudragit layer 2.219. These values are consistent with other systems developed in this study, which was determined to preserve probiotic viability.

C.3.2 Friability of the BMTTS

The friability of the lactose layer of the BMTTS was calculated to be 0.651% with the friability of the eudragit layer calculated to be 0.712%. Both of these values are within the upper limit of 1% as required by the USP and ensure tablet strength and stability during storage.

C.3.3 *In vitro* probiotic release profile of the gastro-resistant BMTTS

The formulated BMTTS when placed in simulated gastric conditions had minimal release of less than 10% upon exposure to simulated gastric fluid at pH 1.2. This can be attributed to the initial swelling of the ovalbumin minitablet and resultant release of probiotic bacteria from beneath the minitablet surface. Upon exposure to simulated intestinal conditions, the effect of enzymatic degradation can be seen with the rapid dissolution of the ovalbumin minitablet with 100% TPC reached within 3 hours (Figure C.2). Upon exposure to simulated colon conditions, the release of probiotic bacteria was significantly less than over the initial 2 hour (less than 15%), due the lag in time taken for the dissolving of the outer polymer layer, combined with the lag seen with bacterial ATP production. The inability of the BMTTS to reach 100% TPC after 8 hours can be attributed to the lack of enzymatic degradation by trypsin in addition to matrix stabilization after erosion at higher pH. This resulted in greater retention of probiotic bacteria within the ovalbumin matrix with cessation in release beginning after 8 hours in simulated colon conditions.

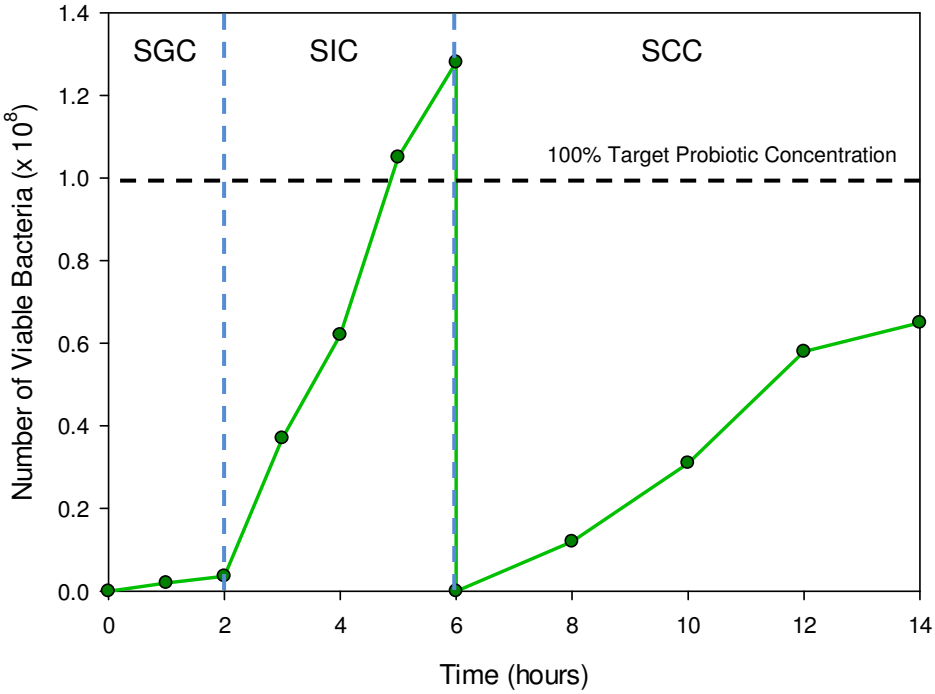

Figure C.2: *In vitro* probiotic release profile of the BMTTS in simulated gastric conditions (SGC), simulated intestinal conditions (SIC) and simulated colon conditions (SCC) (n=3; SD≤ 0.04122 in all cases).

C.4 Concluding Remarks

The BMTTS has been proven to be effective in the protection and site-specific delivery of *L. acidophilus* probiotic to simulated human intestinal and colon conditions. The physicomechanical properties of the BMTTS were also evaluated with results determining a robust tablet formed which would ensure tablet stability over its storage life. All data determined in this evaluation are in correlation with results determined previously in this study confirming the formulated ovalbumin minitablet system can be modified accordingly for the requirements of a delivery system without negatively impacting the viability of the probiotic bacteria entrapped within the ovalbumin matrix.

APPENDIX D

AESC3

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2012/26/05

APPLICANT: **Mr M Govender**

DEPARTMENT: **Pharmacy and Pharmacology**

PROJECT TITLE: ***In vivo* analysis of an oral multi-particulate system derived from ovalbumin for the concurrent delivery of amoxicillin and Lactobacillus acidophilus probiotic in a pig model**

Number and Species

14 Large white pigs

Approval was given for the use of animals for the project described above at an AESC meeting held on **26 June 2012**. This approval remains valid until **30 June 2014**.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Conditions:

- There should be two control pigs in each group.
- Aspirin should not be administered (Heparin is already being administered as an anticoagulant)
- Drug list should include drugs for two anesthesias per individual

Signed: _____ (Chairperson, AESC) Date: 31/7/12.

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 31/7/12

cc: Supervisor:
Director: CAS

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Fax: (011)974-8986

synergyhealth

Gamma Radiation Processing

Certificate Of Gamma Irradiation

This is to certify that

SYNERGY STERILISATION SA (PTY) LTD

Has given an irradiation treatment in accordance with :

. The national and international quality assurance system standards: ISO 9001:2008

. The precision and accuracy of the dosimetry system used is traceable to International Standards

to the following goods (as described by the Manufacturer):

Customer	UNIV OF WITWATERSRAND DEPT OF PI	Customer Order No.	GRN date
Synergy internal order number (Certificate No)	38787 \1	2	8/23/2012
Synergy Stock Code	000 025 TR01	Conforms to the Specified Irradiation Dose of :	
Product description	TRIAL SAMPLE 25KGY.DENS	Minimum :	25 kGy
Quantity of Containers	Mass Of Containers Kg	Maximum :	0 kGy
1	1.00	Routine Dose Reading :	27.6 kGy
		Ref. Dose Mapping :	

Irradiation Completion Date 27/08/2012 Product Released By Quality Department

ISO-9 REV 12